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# HISTOCHEMICAL DETECTION OF GLYCOGEN IN BLOOD CELLS OF THE SOUTHERN ARMYWORM (PRODENIA ERIDANIA) AND IN OTHER TISSUES, ESPECIALLY MIDGUT EPITHELIUM [1]

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## INTRODUCTION

Although the storage of carbohydrate in the form of glycogen is apparently related to the metamorphic processes of holometabolous insects, little is known regarding the processes of intermediary carbohydrate metabolism that occur in insects. Virtually nothing is known as to whether a given status of intermediary carbohydrate metabolism may influence the degree of susceptibility or resistance of an insect to a poison or to an insecticidal procedure. It has been reported (*75*, *91*)[2] that the inclusion of brown sugar or molasses in certain insecticidal sprays or baits may result in an increased kill of certain insects. The added sugar or molasses is believed to cause the poison to be held on the sprayed foliage for a longer time, thereby increasing the amount of poison that the insect ingests. Richardson (*91*) has reported that the addition of brown sugar or molasses increases the solubility of an arsenical insecticide in the spray. In such a case an increase in the carbohydrate content of the gut might cause more poison to be held in solution in the digestive juices of the insect's alimentary tract, whether the carbohydrates were ingested along with or prior to the poison. If this should occur, an increase in the carbohydrate content of the insect's blood or hemolymph (i. e., an alimentary hyperglycemia) might produce a greater solubility of an arsenical poison in the blood, and hence possibly a greater effectiveness of the poison upon the insect's tissues. Ingestion and absorption of sufficient carbohydrate might itself have an injurious or lethal effect upon at least certain species of insects (*19*). Thus, from the standpoint of toxicology as well as physiology, further knowledge of the occurrence, distribution, and metabolism of carbohydrates in insects is required.

In a study of the blood cells of the southern armyworm (*Prodenia eridania* (Cram.)) glycogen was detected in certain of the blood cells and in some of the large midgut epithelial cells that frequently occurred in blood smears from first and second instars. This paper is the report of a qualitative investigation of the occurrence of histo-

[1] Received for publication February 21, 1941.

[2] Italic numbers in parentheses refer to Literature Cited, p. 288. No reference is made to publications appearing after 1939, the year the manuscript was written.

chemically detectable polysaccharide in the blood cells of this insect, and includes similar observations made incidentally upon smeared midgut epithelial cells and cells of various other tissues.

The insects used in this work were larvae, prepupae, pupae, and imagoes that had been reared in cages at about 26.5° C. The larvae were usually reared on fresh green turnip or collard leaves but at times were fed other foods, as indicated under Methods and Results. The imagoes were fed sucrose solution. All the insects used appeared to be normal and healthy animals.

## METHODS

### BLOOD SAMPLING AND SMEARING

To make blood smears in which the cells retain the forms they possessed just prior to sampling and smearing, the insects were immersed in water at 60° C. for 5 minutes. After this heat fixation, the blood was sampled by pricking the larval proleg, the pupal wing case, or the imaginal middorsal thoracic integument (from which the scales had been removed with a brush) with a needle, collecting the blood upon a clean cover slip and, except when the very early instars were used, smearing the blood either with another cover slip or, preferably, with the end of a slide narrowed by the breaking away of its corners. Occasionally blood was sampled from the antennae of an imago. The smears from first and second instars were made immediately to avoid dehydration of the larvae after heat fixation. With these instars and, at times, with third instars, the larva was dragged, with the escaped blood, across the cover-slip surface. In this procedure some epithelial cells from the midgut usually became detached and were included in the blood smear as single cells or as groups of cells. Blood smears free from intestinal cells were obtained when care was taken merely to puncture a proleg, to allow the blood to escape, and then to remove the body without dragging, pressing upon, or otherwise manipulating it. A first- or second-instar blood smear thus obtained consisted of only a tiny drop of blood containing relatively few cells. Tissue smears were prepared by dragging teased tissues across the cover slip.

The results reported here are based on a study of blood smears prepared from more than 300 insects.

### FIXATION AND STAINING

Several fixing procedures were used in preliminary tests to determine one readily applicable to this study. Blood smears from the heat-fixed sixth instars were air-dried and placed immediately in one of the following fixatives: Absolute alcohol, Carnoy's (with chloroform), Neukirch's, Zenker's, Bouin-Allen's, Pasteels and Léonard's, Gendre's, Vastarini-Cresi's, Gulland's alcoholic formol, or 1- or 4-percent aqueous solutions of chromic acid (*67, pp. 226–228; 64, pp. 49, 58, 59, 286–289; 40; 81*). After varying intervals of time the preparations were examined in Lugol's solution (*64, pp. 286–289; 67, pp. 228–230*). Some of the smears from Carnoy's or Neukirch's fixative and from absolute alcohol were stained by Best's carmine method. When chromic acid was used, the smears were fixed for

18 to 24 hours in the 1-percent solution or for 1 hour in the 4-percent solution, and were subsequently stained according to the Bauer method (*64, p. 289; 67, pp. 230–232*). The results of these preliminary tests indicated that Bauer's method (using chromic acid fixation) and fixation in Carnoy's fixative or in absolute alcohol followed by staining in Lugol's solution (wet preparation) or in Best's carmine were the most satisfactory procedures for staining glycogen in the armyworm blood cells.

The Bauer method, with chromic acid fixation, was used most frequently. After chromic acid fixation, the blood or tissue smears were immersed in Schiff's reagent for 25 to 35 minutes, washed in several changes of distilled water acidified with 3 or 4 drops of concentrated sulfurous acid per 100 cc., and counterstained with an aqueous solution of thionin acidified with 2 or 3 drops of concentrated sulfurous acid per 100 cc. A saturated solution of thionin, applied for 10 or 15 minutes, was used to stain some of the smears, but since dye crystals were deposited on the smear and tended to interfere with the observations of the glycogen-stained cells, a two-thirds saturated acidified thionin solution was used on other smears, which were stained for 30 to 60 minutes. Excess counterstain was removed by dipping the smears quickly into distilled water acidified with sulfurous acid. The smears were then dried by blotting on filter paper and warming carefully over a small flame, and were mounted in balsam.

The smears to be stained with iodine were fixed in absolute alcohol for 48 hours or in Carnoy's fluid for 4 to 20 hours (usually about 18 hours), washed with distilled water, covered with a few drops of Lugol's solution and a cover slip, and examined microscopically as wet preparations.

A number of smears were stained by Best's carmine method and used for comparison. They were fixed in absolute alcohol or in Carnoy's fluid. Some were handled according to the usual technique (*64, pp. 287–288*) with Ehrlich's haematoxylin as a counterstain. Other smears were stained as usual with Best's carmine for 10 to 15 minutes and then for about 6 minutes with equal parts of absolute alcohol, Wright's blood stain solution (i. e., the usual alcoholic solution, undiluted), and a 2.5-percent solution of nicotine. The excess stain was removed by flooding the slides with the usual differentiating solution, which was sometimes acidified slightly with oxalic acid to increase the differentiation. Excess differentiating fluid was removed by quickly blotting with filter paper. The slides were dried carefully over a small flame and mounted in balsam.

Smears were also stained by the Wright oxalic acid-nicotine procedure (*115*) and used for comparison. Slightly less satisfactory staining of blood smears from heat-fixed insects (*Prodenia eridania*) was also obtained by the usual Wright's blood-staining procedure. Unless otherwise specified, the Wright oxalic acid-nicotine (W–N–O) procedure was used when Wright staining is indicated. Counterproof smears were fixed in absolute alcohol, treated with saliva at about 35° C. for 5 hours, and stained with Lugol's solution and by the Bauer method. Others, in accordance with Gendre's (*40*) suggestion, were stained by the mucicarmine method of Mayer and used as counter-

proofs to the Bauer method. Negative counterproof results indicated that the glycogen had been digested by the ptyalin of the saliva, and that the positive Bauer reaction was not caused by mucin.

### IDENTIFICATION OF INSTARS

To identify the different instars used in this investigation, head-capsule measurements were utilized. A larva was considered to be of a given instar when its head-capsule measurement (width) approximated the mean value of head-capsule measurements for that instar as determined by Mayer and Babers,[3] whose data show that the southern armyworms reared at this laboratory develop through six instars. Generally, the mature larva stops eating a short time before entering the ground. The age within a given instar was determined, in some cases, by isolating a larva of known instar, noting when it molted, and utilizing it at a determined subsequent hour. The age of a first instar was determined from the time of hatching. Similarly, ages of pupae and adults were determined, respectively, from the times of pupation and emergence.

### STARVATION AND FEEDING

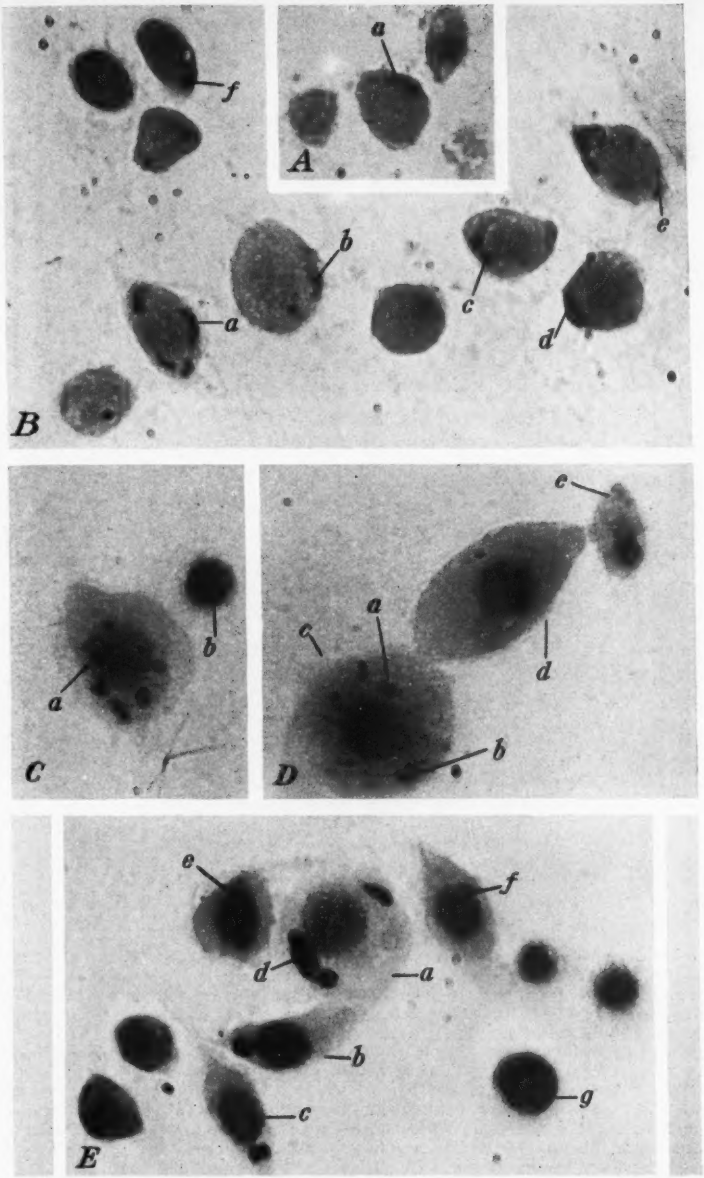
In starvation and feeding experiments upon newly hatched larvae, insects that hatched at the same time were separated into six groups. One group was starved. Each of the other groups was fed one of the following diets: (1) Turnip leaves, (2) gelatin gel, (3) gelatin gel containing glucose, (4) cornstarch paste, and (5) cornstarch paste containing glucose. A mold soon developed on the gelatin, with or without glucose, which was fed to the larvae in the form of a gelled layer covering the insides of the beakers holding the larvae. The cornstarch diets were supplied as a thick paste prepared by boiling.

Sixth instars were also starved or fed individually the following diets: (1) Turnip leaves, (2) collard leaves, (3) white cabbage leaves, (4) green soybean leaves, (5) yellowed soybean leaves, (6) cornstarch paste containing glucose, and (7) cornstarch-glucose paste in turnip-leaf sandwiches. In these experiments the foods were supplied to the larvae qualitatively, but in general the ratio of glucose to cornstarch was about 1 to 10. In other experiments larvae were fed larger amounts of glucose (up to about 1 part to 2 parts of boiled cornstarch). After having access to cornstarch-glucose-turnip leaf sandwiches for a given length of time (usually about 16.5 hours), and an interval of 2 to 3 hours' starvation, some of these larvae were ligatured.[4] Ligatured and unligatured larvae were then fed cornstarch-turnip leaf sandwiches.

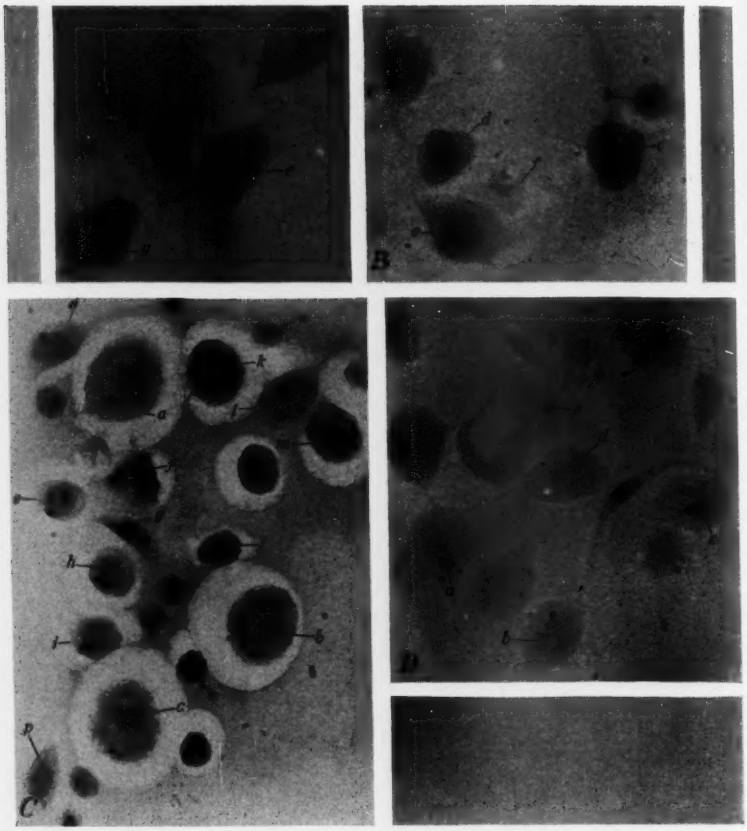
The periods of starvation and feeding of the young and the mature larvae differed. As a rule the newly hatched larvae were starved until dead or were fed for about 3 days. The mature larvae that were to receive the special diets were kept without food for about 30 hours and were then fed for about 4 days. The starved mature larvae were kept without food up to about 70 hours. At the end of the starvation and feeding periods, individual larvae were used for glycogen detection.

[3] MAYER, E. L., and BABERS, F. H. HEAD CAPSULE MEASUREMENTS OF SOUTHERN ARMYWORM LARVAE (PRODENIA ERIDANIA). [To be published.]

[4] These larvae served as controls in experiments on the effects of administered poisons on blood-cell glycogen.




Blood cells from larvae of *Prodenia eridania* that had been fed turnip leaves: *A*, Second instar; *B*, fourth instar; *C*, *D*, and *E*, fifth instars. Glycogen inclusions are shown in *A* at *a;* in *B* at *a–f;* in *C* in a plasmatocyte, *a*, but not in the small cell *b*, which was more deeply stained with thionin; in *D* at *a* and *b* in the plasmatocyte *c*, but not in the cystocyte *d* or in the cell *e;* in *E* conspicuously in the plasmatocytes *a*, *b*, and *c*, as at *d*, less conspicuously in the plasmatocytes at *e* and *f*, but not in the other cells. In *E* note the polar locations of the glycogen inclusions in the fusiform plasmatocytes *b* and *c;* a metaphase is shown at *g*. Blood smears, Bauer technique; oil immersion, 10× ocular, 93× objective.




Blood cells from larvae of *Prodenia eridania: A* and *B*, Sixth instars fed turnip, collard, and cabbage leaves; *C*, a greatly tapered prepupa reared on turnip leaves; *D*, a day-old pupa reared on turnip leaves. In *A* glycogen inclusions are shown at *a* and *b* in plasmatocyte *c* and at *d* in plasmatocyte *e*, but not in plasmatocytes *f* and *g* or in cell *i*, a type that has not been observed to contain glycogen; a nuclear band, *h*, is visible in *g*. In *B* glycogen inclusions occur in plasmatocytes *a*, *b*, and *c*, but not in plasmatocytes *d* and *e* or in cell *f*, which is the same type as cell *i* in *A*. In *C* granular glycogen inclusions are shown in the cystocytes *a*, *b*, and *c*, and larger inclusions in plasmatocytes *d–m*, particularly in *k*, *l*, and *m*; glycogen also appears in some of the small cells, as at *n* and *o*; the nucleus of a plasmatocyte appears banded at *p*. In *D* glycogen inclusions are shown at *a–c*. Blood smears, Bauer technique; oil immersion, 10× ocular, 93× objective.

### THE GLYCOGEN COUNT AND THE GLYCOGEN INDEX

A glycogen count was obtained from a blood smear by taking a certain number of cells at random and classifying each cell according to whether it contained one, two, three, or four or more glycogen inclusions. All the cells observed in each of a series of microscopic fields, visible with high dry magnification (about 660×), were counted until a total of 400 cells had been classified. From these figures the percentages of the blood-cell population of the smear that contained the various numbers of glycogen inclusions and the total percentage of cells that contained glycogen were calculated. The percentages of cells containing the various numbers of glycogen inclusions constitute what is here called the glycogen count. The total percentage of cells that contain glycogen constitutes the glycogen index.

## RESULTS

### BLOOD-CELL GLYCOGEN IN INSECTS FED TURNIP LEAVES

Glycogen was not detected in the blood cells of newly hatched larvae by any of the methods used. In blood cells of larvae fed turnip leaves glycogen was first observed 18.5 hours after hatching and then in only one cell in only one larva. In one other larva glycogen was detected in several blood cells at some time between 23 and 39.5 hours after hatching. Blood-cell glycogen was usually not observed in older first instars or in second instars. When glycogen did occur, it was seen in very few blood cells of the first instars and in more cells of the second instars.

Plate 1, *A*, shows a glycogen-containing blood cell from a second instar. The youngest larva to show glycogen in a considerable number of blood cells was a second instar just after molting. In general, later instars that possessed blood-cell glycogen tended to show it in increasing numbers of cells with each succeeding instar (fig. 3). Plates 1, *B–D*, and 2, *A* and *B*, show some glycogen-containing blood cells from fourth, fifth, and sixth instars. Relatively large numbers of blood cells contained glycogen in sixth instars, prepupae, and the very young pupae that did exhibit blood-cell glycogen. In general, blood-cell glycogen tended toward a maximum at the prepupal stage of development, as shown by the variation of glycogen index during larval development and metamorphosis (fig. 3). During metamorphosis the blood-cell glycogen decreased until in later pupal life it reached a minimum, and sometimes seemed to disappear entirely. Plate 2, *C* and *D*, shows glycogen-containing blood cells from a greatly tapered prepupa and a day-old pupa. The decrease of pupal blood-cell glycogen was associated with a marked decrease in the number of cells that appeared in the blood smears. In the smears from old pupae it was frequently difficult to find any blood cells; the few that were found usually contained little or no glycogen. In the imago blood-cell glycogen was rarely observed.

Individual differences in the occurrence of blood-cell glycogen were large in all the instars, particularly in the earlier instars and among individuals from different batches of larvae. No blood-cell glycogen could be detected in some larvae.

### GLYCOGEN IN MIDGUT EPITHELIAL CELLS OF LARVAE FED TURNIP LEAVES

No glycogen was observed in the midgut epithelial cells of newly hatched larvae. Midgut-cell glycogen was observed earliest in a single turnip-leaf-fed larva 6 hours after hatching. It was also observed in several first instars 11, 24, 26, 28.5, 39, and 47 hours old, but not in other larvae of these ages. Plate 3, *A* and *B*, shows glycogen in midgut epithelial cells from a first instar. In this instar the glycogen in these cells generally appeared earlier after hatching and more frequently than did glycogen in the blood cells.

Glycogen also occurred in the midgut epithelial cells of the later instars, although individual differences, as indicated by the smeared gut cells, were quite marked. Plate 4, *A–C*, shows glycogen in some midgut epithelial cells from a third and a sixth instar. As in the occurrence of blood-cell glycogen, some individual insects appeared to have considerable midgut-cell glycogen, whereas others possessed none. In none of the larvae did glycogen appear in all the smeared epithelial cells.

### EFFECTS OF STARVING LARVAE AND OF FEEDING THEM DIFFERENT FOODS

#### Newly Hatched Larvae

When newly hatched larvae were starved, no glycogen was observed in either their blood cells or their midgut epithelial cells, even up to the time of death, which was usually not more than 48 hours after hatching. In plate 3, *C*, are shown midgut epithelial cells from a starved first instar.

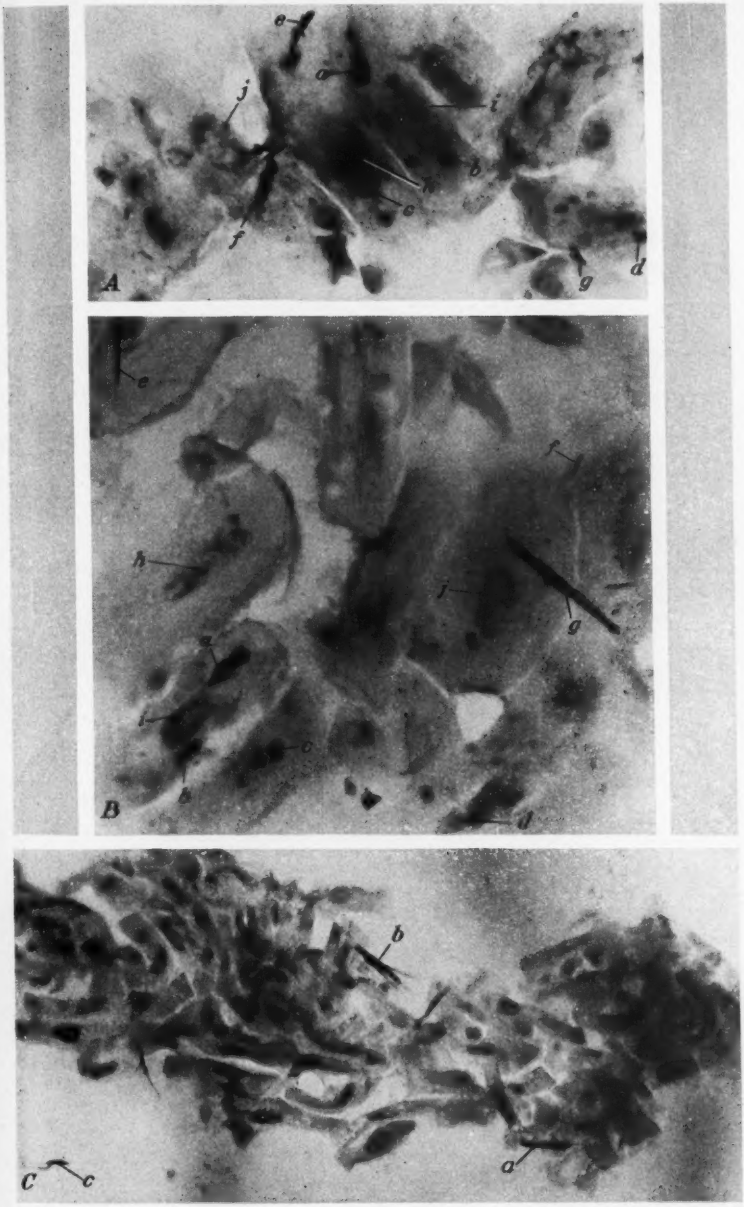
As has been stated, midgut-cell glycogen was observed in some of the first instars fed turnip leaves but not in others, and blood-cell glycogen was observed in only one or two of these larvae and then only in one or two cells in a smear.

Gut-cell glycogen was observed in some larvae fed cornstarch or cornstarch plus glucose, but no blood-cell glycogen was observed in any of these larvae. No gut-cell or blood-cell glycogen was observed in any of the larvae fed gelatin (plus the mold that developed on the gelatin in the course of the experiment). Midgut-cell glycogen was observed in some of the larvae fed gelatin-glucose (plus the developed mold), but blood-cell glycogen was not detected.

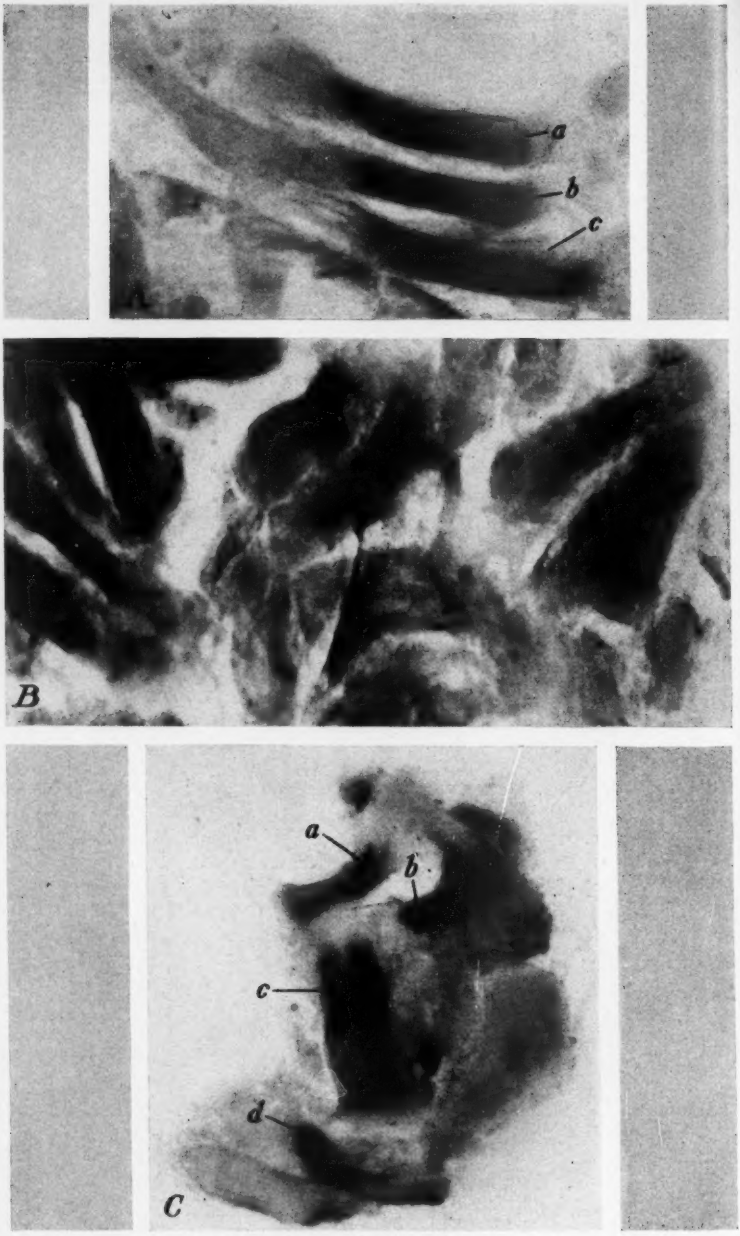
#### Sixth Instars

When sixth instars were taken from diets of turnip and collard leaves and starved for different periods up to 70 hours, glycogen was observed in fewer blood cells the longer the period of starvation until, at about 24 to 40 hours, depending upon the individual insect, it was no longer detected.

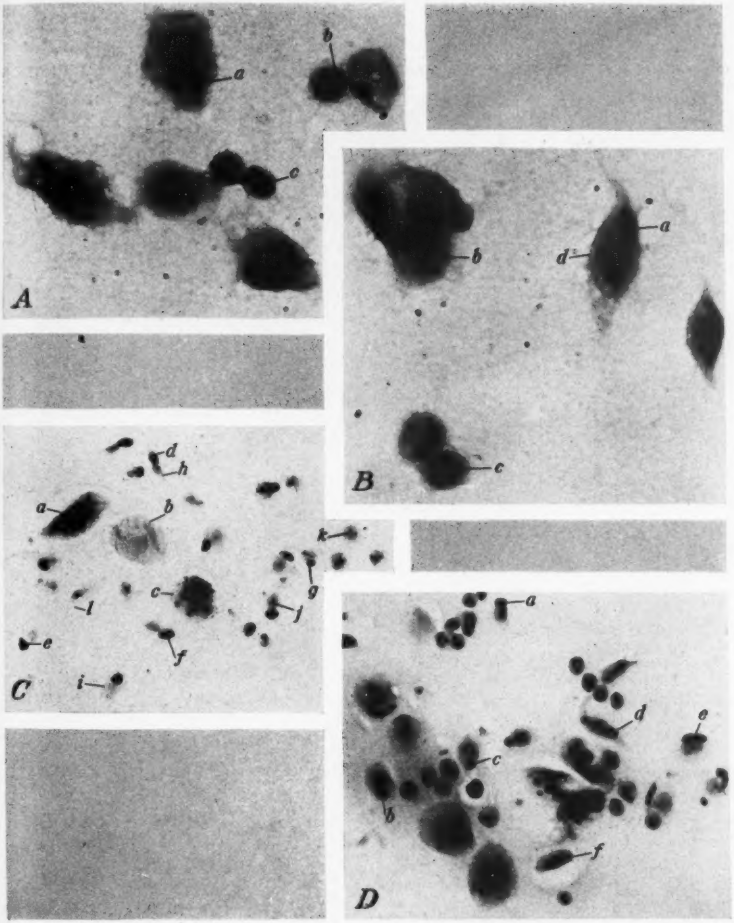
Sixth instars reared on turnip leaves appeared to have more blood-cell glycogen than those reared on collard leaves or, especially, on white cabbage leaves. In larvae fed soybean leaves blood-cell glycogen appeared to be variable, but in general it seemed to be greater when green than when yellowed leaves were fed. Blood-cell glycogen increased when cornstarch-glucose paste, alone or in turnip-leaf sandwiches, was fed to larvae reared on turnip, collard, or white cabbage leaves. Larvae that were fed the smaller quantities of glu-




Midgut epithelial cells of first instars of *Prodenia eridania:* *A* and *B*, Larvae previously fed turnip leaves; *C*, a starved larva 48 to 64.5 hours old. Glycogen inclusions, as *a–d*, are visible in *A* and *B*, but not in *C*. Thionin crystals are shown at *e*, *f*, and *g* in *A* and at *a*, *b*, and *c* in *C*; and nuclei are visible at *h*, *i*, and *j* in *A* and *B*. Tissue smears, Bauer technique; *A* and *B*, oil immersion, 15× ocular, 93× objective, both from the same smear; *C*, dry, 10× ocular, 45× objective.




Midgut epithelial cells of third and sixth instars of *Prodenia cridania: A* and *B*, Third instars reared on turnip leaves; *C*, sixth instars reared on turnip, collard, and cabbage leaves. Glycogen inclusions are shown in cells *a*, *b*, and *c* in *A;* in many of the cells in *B;* and in *C*, those at *a–d* having polar locations. Nuclei are scarcely visible. Tissue smears, Bauer technique; dry, 10× ocular, 45× objective. *C* is from the same larva that yielded the blood smear shown in plate 2, *A*.




Blood cells from sixth instars of *Prodenia eridania* that had been fed turnip-leaf cornstarch sandwiches containing glucose: *A* and *B* from a larva that had eaten a sandwich containing less glucose than in the sandwich eaten by the larva represented in *C* and *D*. In *A* and *B* glycogen appears in all the cells shown, though it is seen more clearly in some, as at *a*, than in others, as at *b* and *c*; the inclusions of the fusiform plasmatocyte *B*. *d*, have approximate polar locations. *C* shows especially well the inclusions in the cystocytes *a*, *b*, and *c*; those of the other cells, as at *d–g*, appear as black areas, but the cell bodies, as at *h–k*, appear very faint; an oenocytoid appeals at *l*. *D* shows glycogen inclusions, as at *a–e*, and an oenocytoid at *f*; the cell bodies are more distinct than in *C*; most of the cells contain glycogen inclusions, but not the oenocytoid *f*. Blood smears, Bauer technique; *A* and *B*, oil immersion, 10× ocular, 93× objective; *C* and *D*, dry, 10× ocular, 45× objective.

Midgut epithelial cells from a sixth instar of *Prodenia eridania* fed turnip leaf-cornstarch sandwich containing much glucose. Glycogen inclusions are shown in most of the cells, as in the parts lettered *a–k;* the inclusions at *e* and *f* are intracellular, although the enclosing cytoplasm is hardly visible in the photomicrograph. Tissue smear, Bauer technique; dry, 10× ocular, 45× objective.

cose (pl. 5, *A* and *B*) usually possessed glycogen in fewer blood cells than did the larvae receiving the larger quantities (*C* and *D*). In one of the larvae that ingested the larger amounts of glucose approximately 85 percent of the blood cells contained glycogen. Some of the cells from these larvae contained numerous and often relatively large glycogen inclusions. In general, it appeared that the greater the amount of glucose in the food and the longer the time of ingestion, the greater was the amount of blood-cell glycogen.

The few observations made also indicated that the feeding of large amounts of glucose in cornstarch-turnip leaf sandwiches to sixth instars increased their midgut-epithelial-cell glycogen (pl. 6).

### KINDS OF BLOOD AND MIDGUT EPITHELIAL CELLS CONTAINING GLYCOGEN

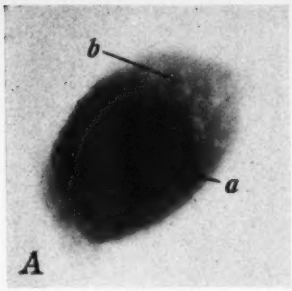
In none of the blood smears, whether stained by the Bauer, the Best, or the Lugol method, were all the different types of blood cells identified with certainty. No attempt will be made in this paper to present a complete classification of the blood cells of the southern armyworm, since this subject is being investigated as a separate study. The blood-cell types mentioned here are described and named in accordance with a tentative classification.

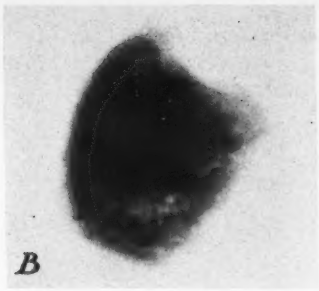
When larvae were reared on turnip or collard leaves, only a part of their smeared blood cells were observed to contain glycogen. In the glycogen-stained smears from first, second, or third instars the identification of blood-cell types was particularly difficult, and in some was impossible. In smears from the later instars, however, especially the fifth and sixth, and from the prepupal and early pupal stages, the blood-cell types, here called plasmatocytes and cystocytes, have been definitely identified among the glycogen-containing cells. These cell types will be described here briefly, in terms of their appearance when obtained from insects fixed in water at 60° C. for 5 minutes and stained with Wright's blood stain.

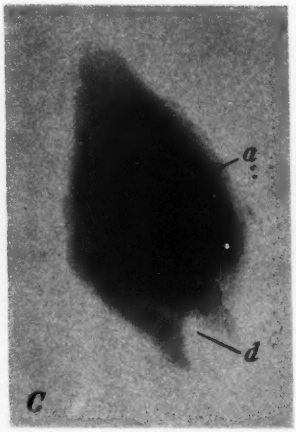
The plasmatocytes are flattened blood cells (pl. 7, *D*) with outlines that may be markedly fusiform (pls. 7, *D* and *E*; 8, *D*), fusiformoid (pl. 7, *A*)—i. e., tending to be fusiform but without definitely pointed spindle ends—ovoid, round, or, at times, polymorphic (pl. 7, *B*). The nucleus is eosinophilic and punctate (pl. 8, *B*), has well-defined chromatin granules, and frequently has suggestions of chromatin threads. It usually contains several bodies that are considered to be nucleoli. The nuclear structure may be obscured by overlying cytoplasm (pl. 7, *A* and *B*), but there is much variation in the degree to which this may occur. The nuclear outline varies from nearly round (pls. 7, *E*; 8, *A–C*) to an elongated oval (pl. 8, *D*), but most frequently it is moderately ovoid. Often the nucleus has a grooved or notched appearance (pl. 8, *C*). A band of basophilic cytoplasm frequently stretches across the visible surface of the nucleus (pl. 8, *A*, *B*, and *D*), and in some cases has been interpreted to be basophilic cytoplasm in the nuclear groove. The cytoplasm contains a poorly developed to very well developed vacuolization (pl. 7, *A*). When poorly developed, the cytoplasmic vacuolization tends to consist of a few irregular, colorless vacuolar regions of different sizes, which may appear to be disconnected or irregularly channeled, or both. The poorly developed vacuolization has been observed to predominate in

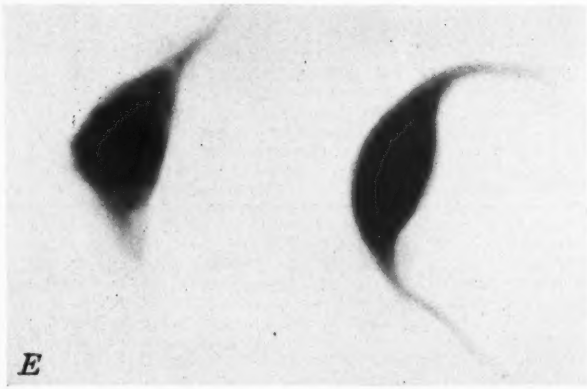
the plasmatocytes of the earlier instars. When the vacuolization is very well developed, the cytoplasm appears to be filled with many small, uniformly distributed, colorless vacuoles, not very distinct individually. Well-developed vacuolization has been observed chiefly in cells from the late instars, particularly the sixth. Most of the observed plasmatocytes of the older larvae, however, exhibited cytoplasmic vacuolizations intermediate between these two extremes (pls. 7, *B* and *E*). The amount of cytoplasm varies from cell to cell. In general, plasmatocytes with smaller amounts of cytoplasm relative to nuclear size tend to be the smaller cells and to possess the more poorly developed vacuolization, whereas plasmatocytes with larger amounts of cytoplasm relative to the nucleus tend to be the larger cells and to possess the better developed vacuolization; exceptions to this rule also occur. Frequently the cytoplasm of the plasmatocyte also contains one or more gross colorless inclusions (pl. 7, *C*), which lie in the finely vacuolized cytoplasm and often are located toward the spindle ends of the fusiform cell. Many plasmatocytes from normally fed larvae do not exhibit these large vacuoles, but those that do may contain one or perhaps two large vacuoles, and occasionally three or more. The plasmatocytes lie on their flat sides in blood smears (pl. 7, *D*). Their nuclei are flattened bodies, and it is probable that the larger of the cytoplasmic vacuoles are also somewhat flattened. The cell peripheries of the plasmatocytes vary in distinctness and often tend to be very indistinct (pls. 7 and 8). The plasmatocyte has not been observed to possess a definitely visible cellular membrane. The surface layer of the cytoplasm seems to be a sort of labile ectoplasm, different from the vacuolized endoplasm, especially in cells that have begun to undergo or have undergone a change in form. Observations upon blood from mature larvae, untreated or incompletely treated by heat, showed that the plasmatocytes readily undergo passive-active transformations (*115*). The plasmatocytes have been observed in the various instars, the prepupa, the pupa, and the adult of the southern armyworm. As indicated by blood smears, they appeared to attain an optimum of cytoplasmic development in the mature larva or prepupa and to decrease in numbers in the pupa.

The cystocytes (pl. 9) are also slightly flattened but are much thicker than the plasmatocytes. Their outlines may also be fusiform, fusiformoid, ovoid, round, or polymorphic. The nucleus is usually eosinophilic but sometimes appears to be basophilic; it is more or less punctate, is usually ovoid to round, and is frequently much obscured or completely hidden by the cytoplasmic inclusions. The cytoplasm is basophilic, but contains large eosinophilic, saclike inclusions, to which the term "cyst" is here applied (pl. 9, *b* and *c*). At times the perinuclear cytoplasm seems to cloak the nucleus with a spurious basophilia. The main body of the fully developed fusiform cystocyte may become bloated with eosinophilic cysts, whereas the spindle terminations of the cell remain thin, lightly basophilic, and completely free of cysts. Sometimes, however, it is difficult to detect the spindle ends of these cells. The number of cysts and the degree of eosinophilia in a cell vary greatly. The cystocytes do not exhibit distinct cellular membranes, but, like the plasmatocytes, appear to possess a sort of labile ectoplasm, which can be more distinctly observed in cells undergoing or having undergone form changes.

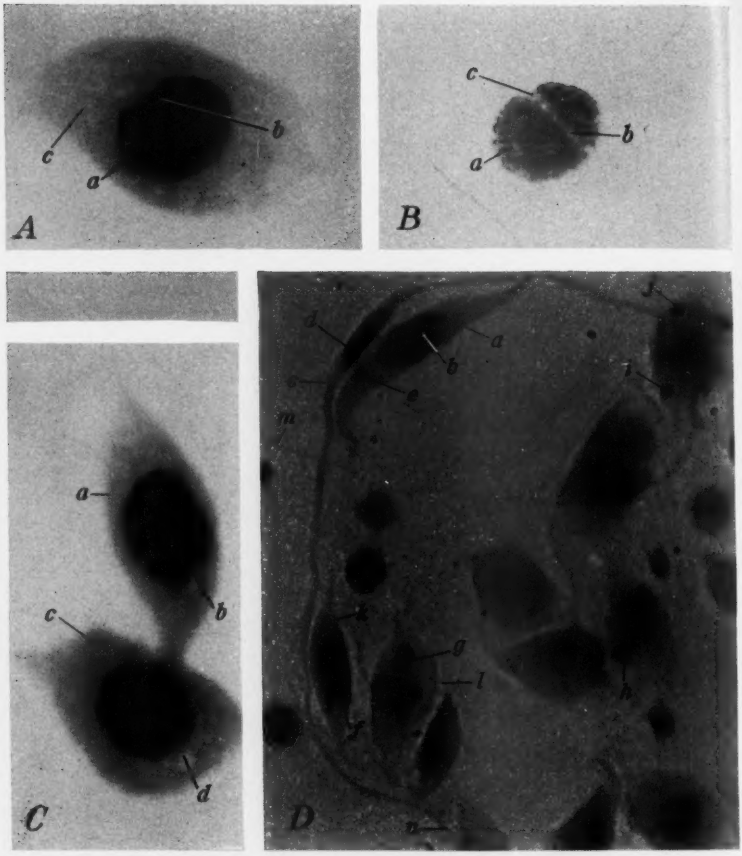


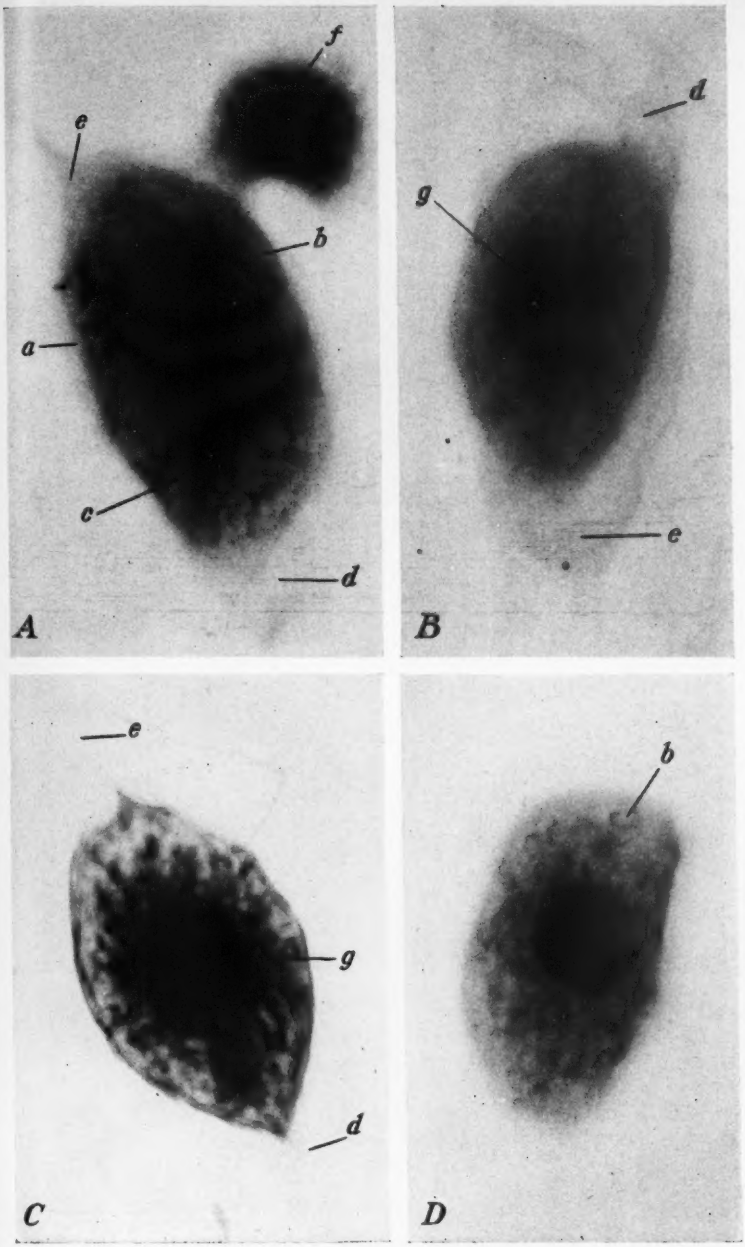





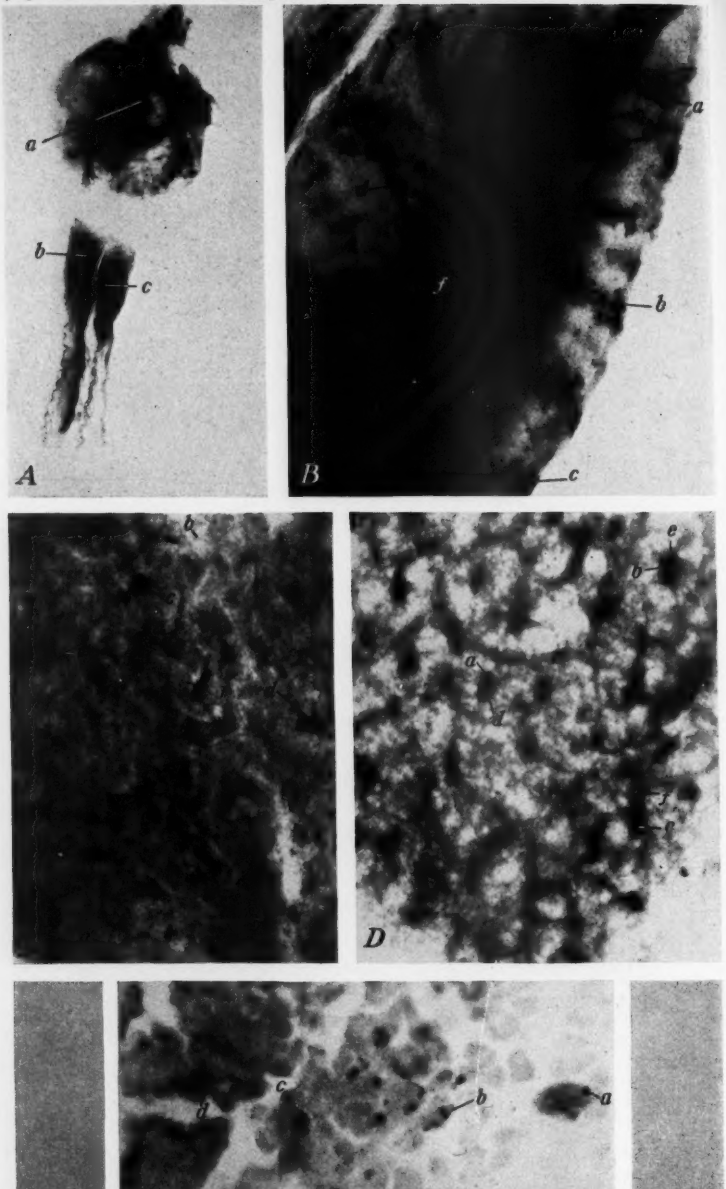

Plasmatocytes, showing finely vacuolized cytoplasm: *A*, Fusiformoid plasmatocyte; *B*, polymorphic plasmatocyte; *C*, *D*, *E*, fusiform plasmatocytes. In each case the vacuolized cytoplasm, as *b* in *A*, tends to obscure the nucleus, as *a* in *A* and *C*, and there is no distinct cell membrane. The plasmatocyte in *C* contains a large cytoplasmic vacuole, *d*, which resembles a glycogen inclusion in form and location; the other plasmatocytes are without gross vacuoles. In *D* the vacuolization is visible only at the ends of the cell; one of the spindle ends, *c*, happens to be folded in such a way as to demonstrate the flattened form of the cell. Blood smears, Wright's technique; oil immersion, 15× ocular, 93× objective.




Plasmatocytes showing banded and notched appearance of nuclei. *A* and *B* represent the same cell. *A* shows the deeply stained nucleus, *a*, across which extends a cytoplasmic band, *b*, and the relatively large area of thinly spread vacuolized cytoplasm, *c;* no distinct cell membrane appears. *B* shows the punctate and banded structure of the nucleus more clearly; *a* is a chromatin mass, *b* is the cytoplasmic band, and *c* is the nuclear notch in which the cytoplasmic band appears to be. *C*, Two plasmatocytes; in one, *a*, the nucleus presents a notch, *b*, which is associated with a band not visible in the illustration; in the other, *c*, is a large cytoplasmic vacuole, *d*, in a finely vacuolized cytoplasm; the cells possess no distinct cell membranes. *D*, Plasmatocyte, *a*, containing a nucleus across which stretches a cytoplasmic band, *b;* the extremely elongated, snakelike cell, *c*, here called a nematocyte, also has a banded nucleus. *d;* glycogen inclusions appear as dark masses in the cytoplasm of a number of cells, as at *e–j;* those in cells *a*, *k*, and *l* occupy polar positions; the ends, *m* and *n*, of the nematocyte, *c*, extend for some distance out of the picture. Blood smears; *A*, *B*, and *C*, Wright's technique, 15× ocular; *D*, Bauer technique, 10× ocular; oil immersion, 93× objective.




Cystocytes showing main body of the cytoplasm loaded with cysts, as *b* and *c*. In *A* the spindle ends, *d* and *e*, of the cystocyte, *a*, contain no cysts, and are very faintly vacuolized and very thin; a plasmatocyte occurs at *f*. In *B* the spindle ends, *d* and *e*, are finely and faintly vacuolized, and the deeply stained punctate nucleus, *g*, is partly obscured by the surrounding cystic cytoplasm. No distinct cell membrane appears in *A* or *B*. In *C* the spindle ends, *d* and *e*, are almost invisible, and the punctate character of the deeply stained nucleus, *g*, is not visible. The cystocyte in *D* illustrates the cystic character of the main body of the cytoplasm, a cyst being especially evident at *b*; the spindle ends of this cell are invisible. The dark lines between the cysts represent basophilic cytoplasm. Blood smears, Wright's technique; oil immersion; 15× ocular, 93× objective.




Various tissues from smears of a sixth-instar *Prodenia eridania* fed turnip-leaf-cornstarch-glucose sandwich: *A*, Ganglion of the ventral nerve cord; large black areas at *a*, *b*, and *c* indicate a positive reaction for polysaccharide. *B*, Labial gland; *a–e* are polysaccharide inclusions, and the dark area, *f*, indicates a position Bauer reaction. *C*, Region of a Malpighian tube, the edge of which appears at *a*; tissue appears to be loaded with polysaccharide, as at *b*, and to contain heavier polysaccharide inclusions, as at *c*, *d*, and *e*. *D*, Part of fat body, showing polysaccharide distributed throughout; the larger inclusions, as at *a*, *b*, and *c*, are in areas of thionin-stained protoplasm, as *d*, *e*, and *f*. *E*, Gonad, showing polysaccharide inclusions, as at *a–d*. Bauer technique, counterstained with thionin; dry: *A*, 10× ocular, 10× objective; *B*, *C*, *D*, and *E*, 10× ocular, 45× objective.

Like the plasmatocyte, the cystocyte is capable of undergoing passive-active transformations. Cystocytes have not been observed in the early instars. They appear later and tend toward a maximum of cytoplasmic development and of numbers at about the prepupal or pupal stage. They decrease to a minimum or completely disappear in the pupal period.

The plasmatocytes and the cystocytes were the chief blood-cell types in which glycogen was observed when larvae were not fed glucose but were reared on turnip leaves or fed turnip or collard leaves for a considerable time. When the turnip or collard leaves were supplemented by a moderate carbohydrate diet (for example, cornstarch or cornstarch with a small amount of glucose) for only a short time, glycogen was also observed occasionally in some of the other types of blood cells. But when larvae were fed the larger amounts of glucose for a sufficiently long time, glycogen could be observed in most of the blood-cell types, insofar as they could be identified in the glycogen-stained smears. For example, glycogen appeared in some of the cells here named "nematocytes" (pl. 8, *D*, *c*) and "polypodocytes" (pl. 13, *B*, *g*). Certain types of blood cells, among which were the oenocytoids (pl. 5, *C*, *l*, and *D*, *f*), were usually found to show no glycogen.

Glycogen inclusions have been observed in mitotically dividing blood cells, apparently distributed to the two prospective daughter cells in unequal amounts. Glycogen was found also in some proleucocytes.

The smeared midgut epithelial cells were frequently recognized as being either columnar cells or goblet cells (*114*). Glycogen was observed in many but not in all of the columnar cells (pls. 3, *A* and *B;* 4, *A–C;* 6) in a given smear.

### INTRACELLULAR LOCATION AND APPEARANCE OF GLYCOGEN IN BLOOD AND MIDGUT EPITHELIAL CELLS

The glycogen inclusions of the blood cells appeared in the form of small granules, rounded masses of various sizes and shapes, and irregular masses of different sizes (pls. 1, 2, and 5). In certain cells from glucose-fed larvae the cytoplasm had a more or less diffuse glycogen stain, and such cells often contained more deeply stained inclusions. Glycogen inclusions were not observed in the nuclei of the blood cells.

The glycogen inclusions in plasmatocytes from insects reared on turnip leaves usually consisted of one or two round to ovoid masses sometimes located one at each pole of the cell (pl. 1, *E*, and 2, *A*). They were similar in location, size, and shape to the large, colorless polar vacuoles in the plasmatocytes of Wright-stained smears (pl. 7, *C*). Sometimes a number of glycogen inclusions were observed in a single cell (pl. 1, *B–E;* 2, *C*), particularly in the prepupa. Greater numbers were observed in some of the plasmatocytes from the glucose-fed larvae, appearing as rounded, ovoid, or somewhat irregular masses varying from the size of a granule to that of a nucleus. An inclusion occurring singly in a cell was also observed to lie anywhere in this range, although usually it was larger than a granule (pl. 5, *C* and *D*).

The plasmatocytes sometimes contained glycogen inclusions in which the glycogen reaction appeared faint. Sometimes the red color (Bau-

er's stain) was localized at the periphery of the inclusion, the center appearing pale or colorless and the entire inclusion having a vacuolar aspect.

The glycogen inclusions of the cystocytes also appeared to vary with the glucose intake. Frequently blood smears contained glycogen in the plasmatocytes, though practically none were observed in the cystocytes. When glycogen inclusions were observed in the cystocytes, they usually appeared as several small granules distributed irregularly in the cytoplasm, as many small granules scattered throughout the cytoplasm, or as some condition of granule occurrence between these two extremes (pls. 2, *C;* 5, *C* and *D*). Sometimes cystocytes containing a number of the small glycogen granules also contained one or more larger, irregular glycogen inclusions (pl. 5, *C* and *D*).

In the smeared columnar epithelial cells of the midgut, glycogen inclusions appeared sometimes as irregular patches between the nucleus and the end of the cell (pls. 4, *A* and *C;* 6), at other times as masses located in other regions of the cytoplasm. These glycogen inclusions were frequently elongated in the direction of the long axis of the cell. No glycogen was observed in the nuclei of these cells.

### THE GLYCOGEN COUNT AND THE GLYCOGEN INDEX

Glycogen counts were made from a number of blood smears obtained from larvae fed turnip leaves, collard leaves, and turnip leaf-cornstarch sandwiches containing different amounts of glucose. The larvae were fed for different lengths of time. Some of these larvae served as nonpoisoned controls in experiments on the effects of poisons on blood-cell glycogen. Some of the control larvae were ligatured by means of a string tied tightly about the body to separate it into approximate anterior two-third and posterior one-third portions; these larvae yielded counts from the fore and hind parts of their ligatured bodies. The period between application of the ligature and sampling of the blood ranged from 1 to 29 hours. Other control larvae were left unligatured. Under these different conditions the blood cells were found to contain various amounts of glycogen. The glycogen counts, however, exhibited similar general characteristics.

The fundamental form of the glycogen count is shown graphically in figure 1, *A*, in which the plotted points are averages of 94 counts from 73 larvae (21 ligatured plus 52 nonligatured) used as controls. This graph shows that on an average, even though counts from larvae fed large amounts of glucose were included, most of the blood cells contained no glycogen inclusions, some contained one inclusion, fewer contained two and still fewer three inclusions, and more cells contained over three inclusions than contained three. The glycogen count thus consists of the following classes: 0, 1, 2, 3, and 3+. The graph represents the general form of the majority of the individual counts. As shown by the glycogen index, the average larva of this group had glycogen in about 31 percent of its cells. In a few instances the 3+ class contained fewer cells than the 3 class. Examination of the corresponding blood smears showed that the cystocytes contained little or no glycogen.

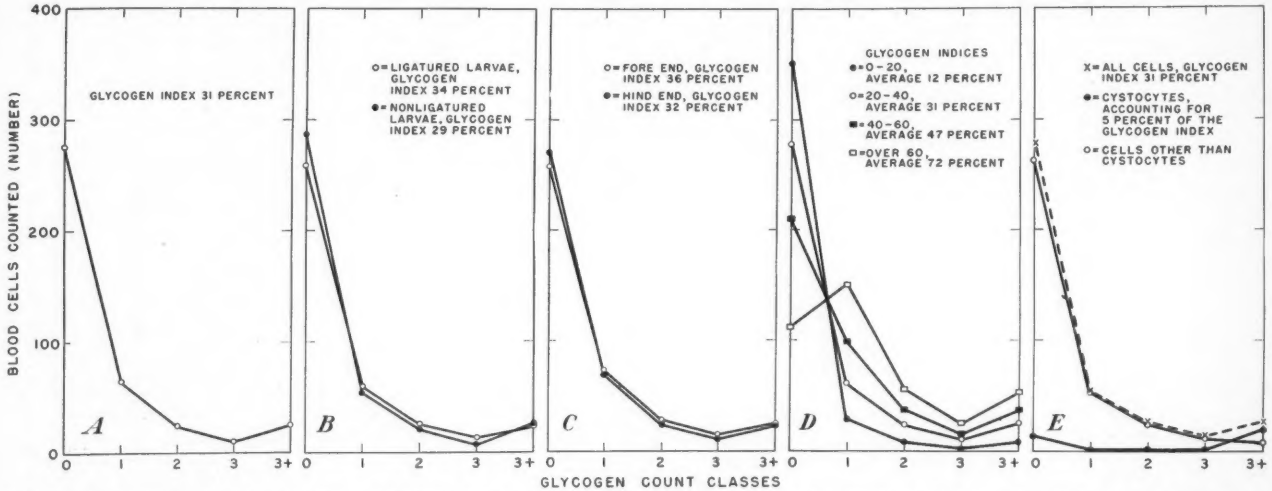



FIGURE 1.—Average glycogen counts and indices for larvae of *Prodenia eridania*: *A*, For 21 ligatured plus 52 nonligatured larvae; *B*, for 21 ligatured and for 52 nonligatured larvae; *C*, for the fore and the hind ends of ligatured larvae; *D*, for ligatured plus nonligatured larvae used as controls, grouped according to four glycogen-index ranges; *E*, for 11 larvae fed turnip leaves, showing separation of cystocytes from other blood cells.

Figure 1, *B*, represents the average glycogen counts for the 21 ligatured and the 52 nonligatured larvae. The two curves are of the form shown in *A*, and differ chiefly in that they have different glycogen indices.

Figure 1, *C*, shows the average glycogen counts from the fore and hind parts of the 21 ligatured larvae. The average fore and hind end values are so similar that they might be represented by a single curve. The average glycogen indices are nearly the same.

In figure 1, *D*, are glycogen counts grouped according to the size of their corresponding glycogen indices. The curves show the way the glycogen count changes with change in index. As the glycogen index increases, the number of cells decreases in class 0 and increases in the other classes, as must happen; furthermore, the cells in class 1 become relatively more numerous than the cells in class 2, which show greater relative increase than the cells in class 3, and class 3+ undergoes a greater relative increase than class 3. When the glycogen index exceeds a certain value (60–70 percent in these experiments), the peak of the curve passes from class 0 to class 1.

Figure 1, *E*, shows the average glycogen count for 11 turnip-leaf-fed larvae. This curve has the general form already indicated in *A*–*D*. Other curves show the effect of counting separately the cystocytes and the cells other than cystocytes. When blood cells exclusive of the cystocytes were counted, the cells of class 3+ were not more but less numerous than those of class 3, the cystocytes were more numerous in class 3+ than in class 3 or classes 2 and 1, and in class 3+ the cystocytes outnumbered the other cells.

Figure 2 shows the frequency distribution of the glycogen indices of the 21 ligatured larvae (fore and hind ends), the 52 nonligatured larvae, and the 73 ligatured plus nonligatured larvae used as controls, with regard to their respective averages of 34, 29, and 31 percent.

Figure 3 shows average glycogen indices of the second to sixth instars, and of the prepupal and pupal stages. The averages are based upon determinations from 5 second, 6 third, 3 fourth, 8 fifth, and 11 sixth instars, 6 prepupae, 11 pupae 1 day old, 3 pupae 2 days old, 2 pupae 3 days old, and 4 pupae 10 days old. Although the number of insects used was not great, figure 3 indicates the manner in which the glycogen index varied with stage of larval development and with metamorphosis. The average index for the sixth instar was 31 percent, and for the prepupa 35 percent. Glycogen was not found in larvae at the time of hatching; therefore the glycogen index was 0 at that time. During larval development the glycogen index increased until it attained a maximum in the prepupa. It rapidly decreased to a very low value during the first few days after pupation, and continued low throughout the rest of pupal life. The few incidental observations made on the adults indicated that the glycogen index of the adult tended to be very low.

### POLYSACCHARIDE IN OTHER TISSUES

Incidental observations were made of a polysaccharide, stained by the Bauer technique, in cells other than those of blood and midgut epithelium. Since the Bauer method gives positive results with polysaccharides other than glycogen, and since only the Bauer method,

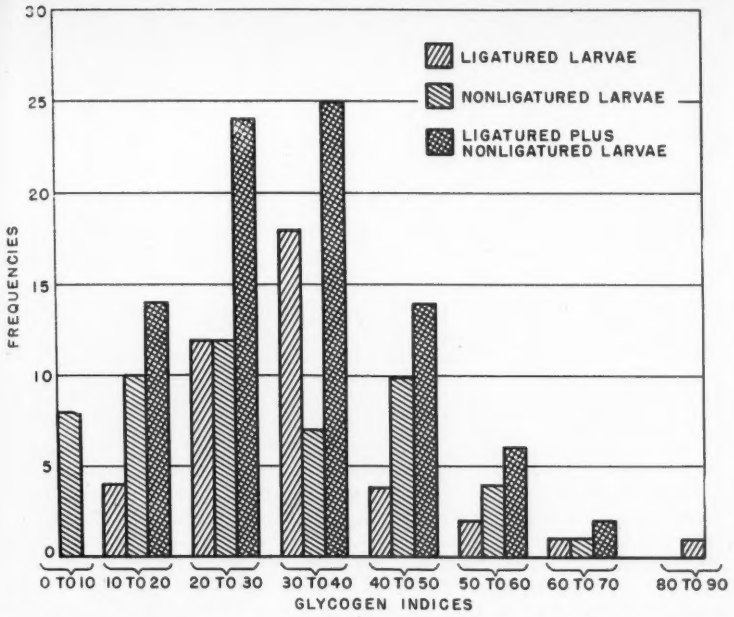



FIGURE 2.—Histogram showing the frequency distribution of 94 glycogen indices from fore ends and hind ends of 21 ligatured larvae, from 52 nonligatured larvae, and from the total 73 ligatured plus nonligatured larvae of *Prodenia eridania* used as controls.

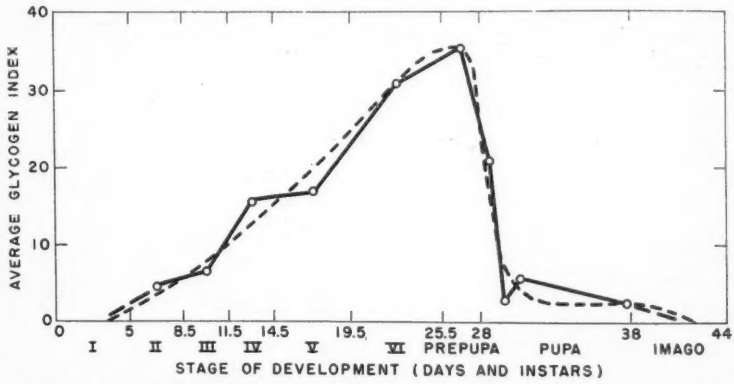



FIGURE 3.—Change of average glycogen index with development of *Prodenia eridania*. Along the abscissa roman numerals represent instars and arabic numerals represent days after hatching. The broken-line curve is hypothetical.

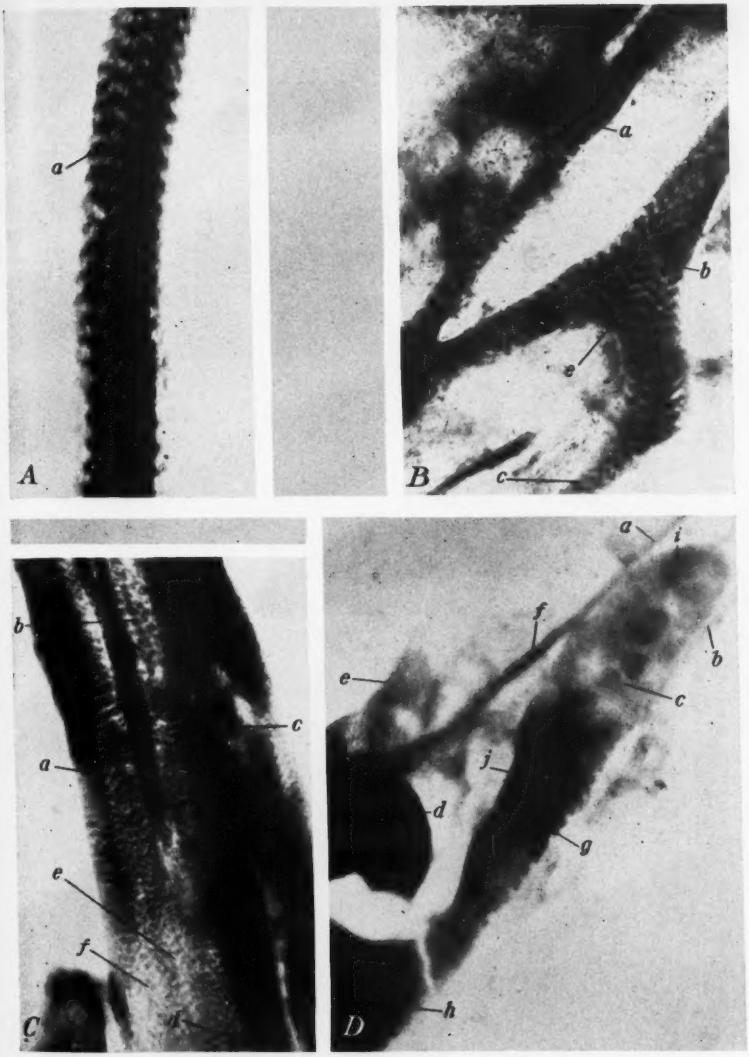
without counterproof, was applied to most of these tissues, the broader term "polysaccharide" is here used except in those cases where the Lugol stain was also applied.

The polysaccharide was detected in the following tissues of a sixth instar that had been fed overnight on a turnip leaf-cornstarch-glucose sandwich, and whose blood-cell glycogen index was 35 percent: Ganglia of the ventral nerve cord, labial glands, Malpighian tubules, fat body, oenocytes, testes, pericardial cells, integument, walls of the foregut and hindgut, midgut epithelial cells, and striated fibers of the body muscles, the gut musculature, the cardiac muscle, and the alary muscles. It was not determined whether the polysaccharide in the ventral nerve ganglia and the interganglionic connectives (pl. 10, *A*) was localized in nerve tissue or in associated nonnerve tissue. The polysaccharide inclusions of the labial-gland cells (pl. 10, *B*) appeared to be numerous, irregular masses of various sizes, many of a coarse granular aspect. The walls of the Malpighian tubes (pl. 10, *C*) contained numerous coarsely granular polysaccharide inclusions, whose relation to the tubular cells was not definitely determined. Similarly, the cytological location of the numerous polysaccharide inclusions of the fat body (pl. 10, *D*) was not definitely determined. The polysaccharide of the striated muscle fibers of the body (pl. 11, *A*), of the fore, mid, and hind parts of the gut (pl. 11, *B*), and of the cardiac muscle (pl. 11, *C*) appeared to occur diffusely, but in addition appeared particularly concentrated at the cross striations, which were seen as deeply stained double lines. The polysaccharide of the alary muscle fibers (pl. 11, *D*) appeared slight but also associated with the cross striations. The oenocytes were opaque, but in places their cytoplasm appeared to be filled with granular polysaccharide inclusions of various sizes and shapes. Polysaccharide was associated with the testes (pl. 10, *E*), but the kinds of sells containing it were not identified; it was not detected in several stages of developing germ cells that were examined. The cytoplasm of the pericardial cells (pl. 11, *D*), contained many very small, granular polysaccharide inclusions. Polysaccharide was also found in the integument, but its cytological location was not determined. The sclerotic membrane of the integument, as well as the sclerotic linings of the fore- and hind-guts, gave a positive reaction with the Bauer technique. Polysaccharide inclusions were not observed in those nuclei whose structure could be sufficiently examined. In the smears of the fat body some of the polysaccharide inclusions were associated with definite masses of basophilic protoplasm (pl. 10, *D*), but whether with nucleoplasm was not determined.

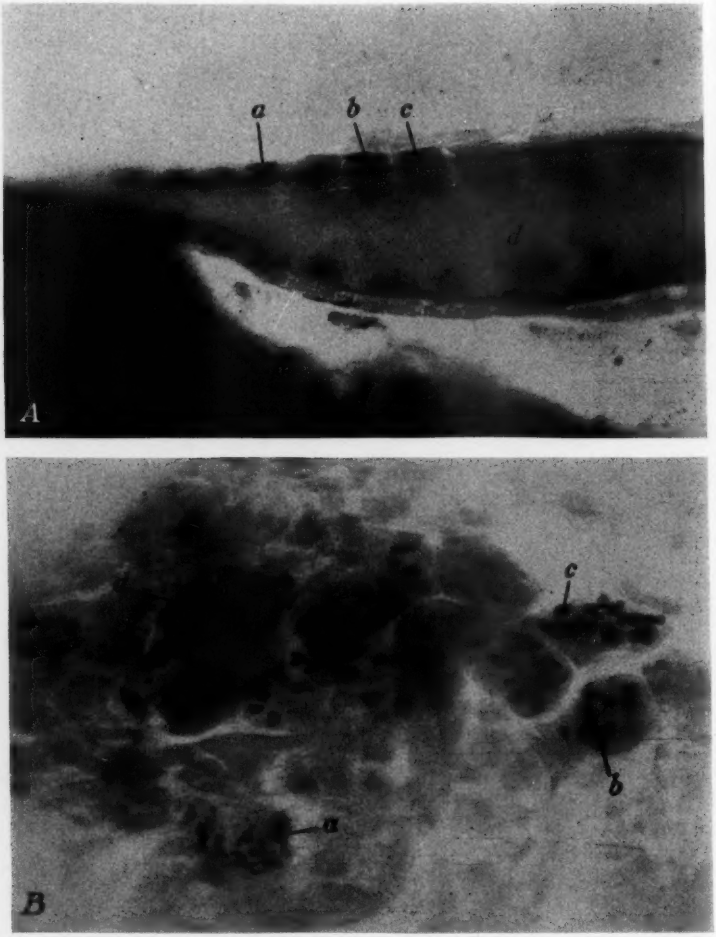
In some of the early instars polysaccharide was found incidentally in the Malpighian tubes, labial glands (pl. 12, *A*), and oenocytes (pl. 12, *B*), when Bauer's technique was employed, and in the labial glands and oenocytes when they were stained with Lugol's solution.

### EFFECTS OF DIFFERENT FIXING AND STAINING METHODS

Although at least traces of glycogen were detected histochemically with nearly all the fixatives mentioned under Methods, preliminary experiments with different fixatives and with the Best and the Lugol staining procedures led to the conclusion that the most satisfactory fixations were obtained with Carnoy's fluid and with absolute alcohol. This is indicated for the Lugol procedure by the glycogen indices given in table 1, which were obtained from two larvae that had fed overnight on glucose-cornstarch-turnip leaf sandwiches.




Different kinds of muscle fibers from smears of a sixth-instar *Prodenia eridania* that had been fed turnip-leaf-cornstarch-glucose sandwich: *A*, Part of body muscle with cross striations, as at *a*; *B*, branched muscle fibers of the foregut, as at *a*, *b*, and *c*, showing cross striations, as at *e*, and bacteria from gut contents as at *d*; *C*, cardiac muscle, with cross striations, as at *a–d*, and fiber boundaries, as at *e* and *f*; *D*, alary muscle fibers, *a* and *b*, with cross striations faintly visible, as at *f*, *g*, and *h*, pericardial cells, *c*, and cardiac muscle, *d*, with cross striations visible at *e*. Polysaccharide appears diffusely throughout the muscle and muscle fibers, and is especially concentrated at the cross striations; in the pericardial cells it appears as small granules distributed in the cytoplasm, which accounts for the dark areas *i* and *j* in *D*. Bauer technique, counterstained with thionin; dry; 10× ocular, 45× objective.




Tissue smears from a 24-hour-old first-instar *Prodenia eridania* fed turnip leaves, showing polysaccharide inclusions: *A*, In cells of a labial gland, *d*, at *a*, *b*, and *c*; *B*, in some faintly stained oenocytes at *a*, *b*, *c*, and *d*. Bauer technique, counterstained with thionin; dry; 10× ocular, 45× objective.

Glycogen indices from Bauer-stained smears of these larvae were 35 percent for larva 1 and 25 percent for larva 2.

TABLE 1.—*Adequacy of different fixatives when used with the Lugol procedure, as indicated by the glycogen index*[1]

| Fixative | Larva 1 | | Larva 2 | | Remarks |
|---|---|---|---|---|---|
| | Time in fixative | Glycogen index | Time in fixative | Glycogen index | |
| | *Hours* | *Percent* | *Hours* | *Percent* | |
| Absolute alcohol (2 changes) | 26 | 35.75 | 29 | 29.25 | Cells shrunken, glycogen inclusions distinct. |
| Carnoy (with chloroform) | 4 | 35.00 | 5 | 26.00 | Cells well fixed, most glycogen inclusions distinct but some so faint as to be questionable. |
| | 26¼ | 39.00 | 28¼ | 33.50 | |
| Neukirch (formol saturated with glucose) | 26 | 3.00 | 29 | ..... | Cells somewhat distorted, glycogen inclusions faint. |
| Zenker | 27 | 23.50 | 27 | 23.50 | Cells shrunken, glycogen inclusions faint but well defined. |
| Bouin | 27½ | ..... | 26½ | ..... | Vacuoles distinct but colorless. |
| Pasteels and Léonard | 1 | 8.00 | 1¼ | 18.25 | Cells distorted; glycogen inclusions, when present, generally faint. |
| | 4½ | 25.25 | 4¾ | 1.50 | |
| | 27 | 13.00 | 28 | ..... | |
| Bouin-Allen | 4¾ | 6.75 | 5½ | 6.25 | Vacuoles distinct but nearly colorless. |
| | 27 | ..... | 26 | ..... | |
| Vastarini-Cresi | 26 | ..... | 28 | 11.50 | Cell fixation fair, glycogen inclusions indistinct and faintly stained. |
| Gendre | 4¼ | 2.75 | 4 | 5.50 | Cells distorted; glycogen inclusions, when present, faintly stained. |
| | 27 | ..... | 26 | ..... | |

[1] No indices were obtained from the smears with extremely poor glycogen fixation.

The glycogen inclusions of the blood cells and midgut epithelial cells were more clearly defined in the thionin-counterstained Bauer smears than in the wet Lugol preparations. Chromic acid fixation involved some shrinkage of either cells or plasma film, or both, as indicated by a space between cell and plasma (pls. 2, *C*; 5, *C* and *D*). Although the thionin counterstain persisted for months, eventually it faded. None of the saliva-treated smears contained glycogen, and none of the smears treated according to the mucicarmine method of Mayer gave positive results, although they were from larva that contained blood-cell glycogen as indicated by the Bauer reaction.

Plates 13, *A*, and 14, *A* and *B*, illustrate the results obtained by combining Best's carmine and the Wright techniques. In cells thus stained the eosinophilic nuclei and basophilic cytoplasms were stained as with the Wright procedure alone and the glycogen inclusions were stained a dark blue. Plate 13, *B*, shows cells stained with Best's carmine and counterstained with Ehrlich's haematoxylin. The glycogen inclusions are stained the usual carmine color.

## DISCUSSION

### OCCURRENCE OF BLOOD-CELL GLYCOGEN IN ANIMALS

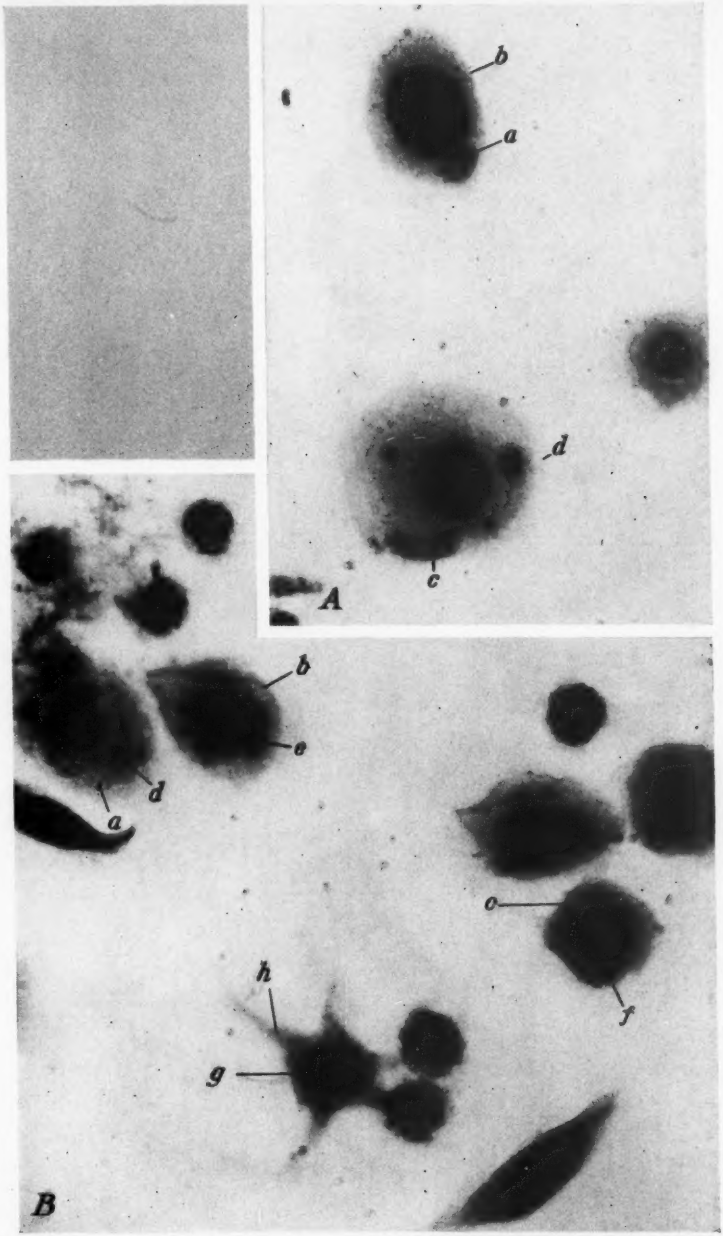
The results described above show that the appearance of glycogen in the blood cells of *Prodenia eridania* is a normal occurrence. The question naturally arises as to whether the southern armyworm is exceptional in this respect or whether such occurrence is general among animals. The authors have found in the literature no paper on insect blood-cell glycogen and only a few papers that deal incidentally with its histochemical detection. Vaney and Maignon (*104*), using the gum-iodine method and the gentian violet method of Lubarsch, re-

ported the histochemical detection of glycogen in leucocytes found associated with adipose cells in the silkworm during metamorphosis; they consider the glycogen not to be of histolytic origin. Rudolfs (*96*), also using iodine and gentian violet staining methods, reported the detection of glycogen in the leucocytes of the adult tent catapillar (*Malacosoma americana* (F.)). De Boissezon (*16*) reported that, using the gum-iodine and Best's carmine methods, he found no glycogen in the amoebocytes of the mosquito *Culex pipiens* L., and Paillot (*79*), using Gendre's method of fixation and the staining methods of Best and Lugol, reported no detectable glycogen in the blood cells of the silkworm.[5] Bogojavlensky (*14*), using Carnoy's fixative and the Best carmine and the Lugol iodine staining methods, found glycogen in the spindle-shaped leucocytes and the proleucocytes of *Carausius morosus* Brunner and, by implication, in seven other species of Orthoptera that were used for comparison; he also found a little glycogen in small, round blood cells, but not in the proleucocytes and spherocytes, of the silkworm and, by implication, of nine other species of Lepidoptera. Ronzoni and Bishop (*92*), using chemical methods, found that in the mature feeding and the spinning honeybee larvae the concentration of blood glycogen was less than 10 mg. percent—i. e., not detectable—and that blood glycogen appeared in greater concentration—i. e., became detectable—only at a later stage, when the cells of the fat body broke down during metamorphosis. Their work did not indicate whether the glycogen occurred in blood cells. Roy (*95*) failed to detect glycogen in the blood of the larva of the wax moth (*Galleria mellonella* (L.)).

Babers (*3*) found that the normal glycogen content of the blood of the sixth-instar southern armyworm was from 1.3 to 4.5 mg. per 100 ml., with an average of approximately 3.3 mg., and that the glycogen content of pooled blood from larvae that had fed overnight on glucose ranged from approximately 13.5 to 33.4 mg. per 100 ml. In one experiment Babers found that a blood sample contained 17.42 mg. of glycogen per 100 ml., of which 16.5 mg. was in the centrifuged cells and only 0.92 in the supernatant plasma, and he suggested that the plasma glycogen may have come from some cells ruptured by centrifuging. Babers' glycogen values were obtained from the blood of insects that had been immersed in water at 60° C. for 1 minute. Thus, blood-cell glycogen is reported to occur in the silkworm, the tent caterpillar, the southern armyworm, *Carausius morosus*, and according to the implications of Bogojavlensky's paper, in a number of other species of Orthoptera and Lepidoptera.

Blood-cell glycogen has also been reported in invertebrates other than insects. Barfurth (*5*) suggested that it probably occurs in gastropods as well as in Crustacea. Creighton (*22*) found glycogen in the blood cells of snails. Biedermann (*12*) stated that glycogen occurs in annelid leucocytes. Its occurrence in various protozoa is indicated by Pflüger (*83*) and Biedermann (*12*), who refer to other literature, and by Giovannola (*41*) and Creighton (*22*).

[5] The present authors found inclusions of Bauer-positive polysaccharide in the blood cells of 3- or 4-day-old first-instar silkworms that had been fed only lettuce leaves. These polysaccharide inclusions appeared similar to the glycogen inclusions of southern armyworm blood cells—i. e., in plasmatocytes—and no doubt were glycogen inclusions. However, blood cells of silkworms that were reared on mulberry leaves and were nearing the end of their larval life showed no positive reaction to the Bauer technique.




Blood cells from a sixth-instar *Prodenia eridania* reared on turnip leaves: *A*, Blood smear stained by combined Best's and Wright's techniques; *B*, blood smear stained by Best's method and counterstained with Ehrlich's haematoxylin. Large glycogen inclusions in *A* are shown at *a* in plasmatocyte *b*, and at *c* and elsewhere in plasmatocyte *d*; in *B* in plasmatocytes *a*, *b*, and *c*, at *d*, *e*, and *f*, respectively; *g* is a polypodocyte, with pseudopodlike extensions of the cytoplasm, as at *h*. Oil immersion; 10× ocular, 93× objective.

*A*, Plasmatocyte from a blood smear of a newly formed pupa, and *B*, midgut cells from a tissue smear of a second instar of *Prodenia eridania*, both stained by combined Best's and Wright's techniques. *A* shows a large glycogen inclusion, *a*, and several small inclusions, as at *b*; in *B* glycogen inclusions appear as black masses, as at *a–f*. *A*, Oil immersion, 10× ocular, 93× objective; *B*. dry, 10× ocular, 45× objective.

Most of the literature on the occurrence of blood-cell glycogen of vertebrates appears to have reference to mammals, particularly to man.[6] Ranvier (*89*) found glycogen in frog leucocytes and, according to Giradin (*42*), was the first investigator to demonstrate glycogen in leucocytes, but Schäfer (*98*) also early referred to glycogen in leucocytes, presumably of man. Barfurth (*5*) failed to find glycogen in either cells or plasma of frog blood and did not obtain decisive results on human blood, but he reported blood-cell glycogen in vertebrate leucocytes. Frerichs (*36*), Gabritschewsky (*38*), Dastre (*23*), Kaminer (*56*), Bakker (*4*), Willstätter and Rohdewald (*113*), Fieschi and Bertola (*33*), Giradin (*42*), Golandas (*43*), Staub and Golandas (*100*), Wallbach (*108*), Unshelm (*103*), Kotschneff (*61*), Havet (*47*), Howell (*54, p. 881*), Downey (*29*), Karsner (*57, pp. 61–63*), and Cowdry (*21*) are some authors who state that glycogen occurs normally in certain leucocytes of man and other mammals (dog, cat, calf, herbivores, horse, rabbit). It would seem that usually glycogen occurs chiefly in the granulocytes, especially the neutrophiles, and to a slight degree or not at all in the lymphocytes and monocytes; see, for example, Giradin (*42*), Downey (*29*), Cowdry (*21*).

According to Dastre (*23*), glycogen was first observed microscopically in pus cells by Kühne (1865) and was shown by other investigators, whose papers Dastre cites, to become more evident microchemically under certain conditions of disease. Fieschi and Bertola (*33*), Giradin (*42*), Unshelm (*103*), and Karsner (*57, pp. 61–63*) also consider leucocytic glycogen in pathological conditions, primarily in the blood of man.

It thus appears from the literature that blood-cell glycogen occurs among protozoa, annelids, mollusks, crustaceans, insects, amphibia, and mammals, including man. Although the observations on mollusks and crustaceans seem to be very few, it is probable that blood-cell glycogen is of general occurrence among animals. This conclusion is in accordance with the concept expressed by Bernard (*8*), Barfurth (*5*), and Dastre (*23*) that glycogen may occur in all or most tissues of all or most animals. The southern armyworm, therefore, is not exceptional in containing glycogen in its circulating blood cells.

### INFLUENCE OF NUTRITIONAL CONDITIONS ON BLOOD-CELL GLYCOGEN

The results of the experiments with newly hatched southern armyworm larvae indicate that the appearance of both blood-cell and gut-cell glycogen is dependent on food intake, particularly that of carbohydrate. With sixth instars the amount contained in the blood cells varies with nutritional conditions, especially the amount of carbohydrate ingested. Thus, prolonged starvation may cause the disappearance of blood-cell glycogen, and the ingestion of relatively large quantities of glucose may cause glycogen inclusions to appear in a large percentage of the larva's blood cells.

Such dependence of blood-cell glycogen on carbohydrate intake may help to explain negative results such as those reported by

[6] A number of the references dealing with glycogen in vertebrate blood cells were kindly supplied by C. N. H. Long, of Yale University.

de Boissezon (*16*) and Paillot (*79*), as well as the marked individual differences in both blood-cell and gut-cell glycogen that were noted in the present investigation. Harnisch (*46*) found no evidence of glycogen resynthesis in *Chironomus* larvae during post-anaerobic recovery, except through the ingestion of food. Kato (*58*) found that more glycogen appeared in silkworm larvae fed immature mulberry leaves than in larvae fed mature leaves. Kato, Miwa, and Negi (*59*) obtained glycogen increase in silkworm larvae upon adding sugar to their diet. Kuwana (*63*) found that glucose ingestion caused an increase in total reducing power of the blood in silkworm larvae and considered an increase of glycogen or of a carbohydrate-protein complex to be involved. Thus, it may be expected that, other conditions remaining the same, an increase in the carbohydrate content of the leaves upon which the larva feeds will result in an increase in insect blood-cell glycogen.

### PROBABLE RELATION OF BLOOD-CELL GLYCOGEN TO BLOOD SUGAR IN INSECTS

Babers (*3*) found that the ingestion by the sixth-instar southern armyworm of turnip leaf smeared with glucose-cornstarch paste was followed quickly by a sudden and relatively large rise in blood sugar, which reached a maximum, then quickly fell, and returned to the normal value in approximately 6 hours. He also found that tissue (exclusive of blood and gut) glycogen also quickly began to rise, but rose more slowly, reached a maximum considerably later, and then descended to, not the initial, but a somewhat higher tissue-glycogen level. He further found that a rise in blood glycogen occurred still later, appearing only after a relatively long period of glucose ingestion, and that practically all the blood glycogen was associated with the centrifuged blood cells.

The histochemical observations of blood-cell glycogen reported in this paper are in agreement with the findings of Babers. It is quite probable that Babers' average glycogen value for normal blood of 3.29 mg. per 100 ml. corresponds to the glycogen index of approximately 31 percent reported here for sixth instars fed collard and turnip leaves; but, whereas Babers obtained a blood-glycogen increase of sixfold to tenfold after overnight feeding of a glucose-starch-turnip leaf diet, the highest glycogen index obtained in the present experiment was about 86 percent, representing only about a threefold increase, under fairly similar conditions. Although this difference might be due to differences in food intake, it might be explained by the fact that the glycogen count and the glycogen index are based, not on the amount of glycogen per cell, but on the number of glycogen inclusions per blood cell, and that microscopic observations show that after ingestion of the carbohydrate diet many of the blood cells contain relatively large glycogen inclusions. The glycogen counts and indices reported here also show that, as the glycogen index increases, more cells enter classes 3 and 3+ of the glycogen count. Thus, it seems that an increase of blood-cell glycogen involves an increase in the number of cells containing glycogen, in the amount of glycogen contained within at least some of the glycogen inclusions, and in the number of glycogen inclusions

per cell. It is also possible that glycogen not detectable histochemically may occur and increase with the carbohydrate intake.

Baber's suggestion that the delayed rise of blood glycogen might be associated with a tendency to rise only after the other tissues had become saturated with glycogen receives support from the present observations on glycogen in the tissues of a sixth instar that had fed overnight on a turnip leaf-cornstarch-glucose sandwich. The glycogen index of this larva was about 35 percent, and its tissue cells, particularly those of its labial gland, Malpighian tubes, striated muscles, pericardial cells, oenocytes, and fat body, had the aspect of being heavily loaded with polysaccharide, as indicated by the Bauer technique. The observations on the newly hatched larvae, which showed that blood-cell glycogen first appears later than gut-cell glycogen, also are consistent with the suggestion mentioned above. Thus, a marked or persistent exogenous hyperglycemia seems to produce an increase in both chemically and histochemically detectable blood-cell glycogen, and, on the assumption that starvation results in a lowering of blood sugar, the results of the starvation experiments imply the existence of an analogous relationship between a marked or persistent starvation hypoglycemia and a decrease of blood-cell glycogen. There is some evidence, as reported, for example, by Staub and Golandas (*100*), that administration of glucose to the mammal causes blood-cell glycogen, but not plasma glycogen, to increase. That a lowering of insect blood sugar might result from starvation is indicated in the literature; see, for example, Sturtevant (*102*), Beutler (*11*), and May (*72*). Demjanowski and Prokofjewa (*26*) reported that the total reducing power of silkworm blood was not dependent on the quantity and quality of the food given the insects. Hemmingsen (*48*) reported a decrease in total reducing power of the blood of several species of insects as a result of starvation, but he considered the decrease not to involve blood sugar. That glucose accounts for only a small part of the total reducing power of insect blood is indicated by Hemmingsen (*48*), Kuwana (*63*), Florkin (*34*), and Babers (*2*), as well as others.

Most of the literature having to do with the relation between sugar and glycogen in insects is concerned with the concentrations of these carbohydrates, not in the blood, but in the whole insect, and will be found summarized by Babers (*3*). The observations and results reported in that literature and those reported here indicate that under certain conditions, chiefly in the pupa, the glycogen content of the insect, including blood-cell glycogen, can decrease in the presence of an elevated blood sugar. This is not contradictory to the statement made above that an exogenous hyperglycemia tends to increase the glycogen content of the blood cells, for the elevated blood sugar of metamorphosis may be considered to be endogenous and to involve other metabolic conditions.

It thus appears that the insect, like the vertebrate, possesses a physiological mechanism whereby gluconeogenesis occurs in the blood cells during alimentary hyperglycemia, and that in insects glycogenolysis occurs during presumptive starvation hypoglycemia and during the endogenous hyperglycemia of metamorphosis.

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## OTHER FACTORS THAT MIGHT AFFECT THE OCCURRENCE OF BLOOD-CELL GLYCOGEN

### Food Factors

In this investigation it was found that when larvae were reared or fed for a sufficiently long time on white cabbage, blood-cell glycogen was not readily demonstrable, many of the larvae apparently having none. MacDonald and Wislicki (*69*) prepared two cabbage extracts that they found affected the blood sugar and liver glycogen when fed to mammals. Their hyperglycemic fraction caused a marked increase in blood sugar in the rabbit, accompanied by glycosuria and a decrease in the glycogen content of the liver. Their hypoglycemic fraction "vegulin" lowered the blood sugar and appeared successfully to replace insulin in the depancreatized dog. It is not impossible that such food factors may affect feeding insects, and that such a hyperglycemic food factor in the cabbage may have been partly or wholly responsible for the relatively slight blood-cell glycogen observed in the cabbage-fed larvae of the experiments reported here. Braun and Rees (*18*), in a review of literature pertinent to this subject, indicate that the question of the occurrence of insulinlike substances in plant extracts is in a very unsettled state.

### Hydrogen-ion Concentration

Babers (*2*) found the pH values of the blood of sixth-instar southern armyworms to range from 6.40 to 6.67, with an average of 6.53. Demjanowski, Galzowa, and Roshdestwenska (*25*) found the hydrogen-ion concentration of the blood of silkworm larvae to decrease at the molts, and to be slightly but persistently higher in the female than in the male. Vaney and Maignon (*104*, *105*) reported that the glycogen content of *Bombyx mori* L. was greater in the female than in the male. Babers (*3*) found the glycogen content of the southern armyworm adult to be persistently higher in the female than in the male. Bernard (*9*) reported that the larva of a fly showed much glycogen, no sugar, and an alkaline reaction of the tissues, the pupa showed sugar and an acid reaction of the tissues, and the adult continued to show both sugar and glycogen. Bernard also reported that the liver glycogen of Crustacea increases at the molt and decreases or disappears between molts. Elias and Weiss (*30*) reported that glycogen in frog eggs decreased when the pH value of the suspension medium dropped. Donnelly (*28*) discussed generally the question of hydrogen-ion concentration and glucose metabolism and concluded that glycogenolysis varies directly with the total acidity of a single tissue or of a whole organism. Other investigators have reported observations that have a bearing on the subject. The hydrogen-ion concentration of the blood, therefore, may be an influential factor in the occurrence of blood-cell glycogen.

### Metabolic Conditions

A number of poorly understood or unrecognized factors that influence the presence of blood-cell glycogen may exist in the insect either continuously or under certain conditions of metabolism. Maignon (*70*) and Vaney and Maignon (*104*) produced asphyxia in *Bombyx mori* by immersing the insect or its tissues in boiled oil and irregularly obtained increases of body sugar. Lesser (*66*) stated that glycogen of the frog is strongly hydrolyzed as a result of anoxybiosis.

Harnisch (*46*) obtained an anaerobic decrease of glycogen in certain tissues of *Chironomus* larvae. Rudolfs (*96*) stated, but gave no supporting data or literature reference, that anesthesia of a living cell inhibits the synthesis of glycogen. That the local metabolic processes in a cell or tissue may influence the amount and the character of the glycogen reserve is indicated by the observation of Bogojavlensky (*15*) that the external lobes of the fat body of the silkworm contained more histochemically detected (Best method) glycogen than did the internal lobes, and that the glycogen of the fat body was less soluble in water than was the glycogen of other tissues. The same thing is indicated by the fact that the vertebrate neutrophiles, but usually not the mononuclears, contain considerable glycogen (*4*, *21*, *29*, *42*), and by the observations herein reported that in the normal southern armyworm the plasmatocytes and the cystocytes tended to exhibit glycogen inclusions more readily than did the other blood-cell types. It is generally recognized that muscular exercise causes a decrease of mammalian liver glycogen (*82*, *p. 94*), and Giradin (*42*) found that in man the number of circulating granular leucocytes that contain iodophilic substance increases at meal times and decreases during exercise after meals, tending to parallel the changes in blood-sugar concentration. As shown in the literature, some of which has already been cited, leucocytic glycogen occurs plentifully in pus cells and appears to vary with different diseases.

In the vertebrate fat metabolism and carbohydrate metabolism are closely related (*82*, *pp. 11, 13, etc.*). The literature reviewed by Babers (*3*) indicates the existence of some kind of an association between fat and carbohydrate metabolism in the insect. Fat inclusions in insect blood cells have been observed by a number of authors (Hollande *50*, Hufnagel *55*, and Rooseboom *93*), and possibly they occur in southern armyworm leucocytes, although in the present work no fat detection was attempted. It is therefore possible that in the insect blood-cell glycogen and blood-cell fat may be related. Karsner (*57*, *pp. 61–63*) states that glycogen is likely to appear in many places where fat occurs, and expresses the opinion that similar factors may influence both fat and glycogen. Insofar as they indicate an exogenous origin of blood-cell glycogen, the observations reported here seem to be in accordance with the view of Vaney and Maignon (*104*), who, disagreeing with Bataillon (*6*), considered that glycogen in the leucocytes and other cells of the metamorphosing silkworm is not of histolytic origin.

Cold tends to decrease liver glycogen in the mammal (*82*, *p. 24*), but it appears that the low temperature reflexly initiates muscular contractions (shivering) and that the glycogen decrease is thus related to exercise. If the armyworm possesses no mechanism analogous to the shivering reflex of the mammal, whereby muscular contractions are produced by cold, low temperature could be expected to tend to maintain its glycogen stores or to decrease their rate of disappearance, except insofar as it would tend to decrease the ingestion of food. Ditman and Weiland (*27*) have shown that a decrease of temperature retards the disappearance of glycogen in the pupa of the corn earworm. The honeybee, however, seems to possess a reflex mechanism whereby muscular contraction increases with decrease in hive temperature (*74*, *80*, *84*). Thus, in such an insect it is quite possible that

cold would tend to cause a reflex lowering of any glycogen reserves, including blood-cell glycogen.

Bernard (*8*) early observed that in the higher animals glycogen is widely distributed in the tissues of the developing organism but becomes relatively localized in certain tissues of the adult, whereas in the invertebrates, which he considered to lack true livers, glycogen appears to be more widely distributed among the various tissues. Karsner (*57, pp. 61–63*), however, referring primarily to man, expressed the opinion that practically any cell in the body may contain glycogen although it may not be microscopically demonstrable. It thus seems that most of the tissues of animals can contain glycogen but differ chiefly with regard to the amount contained. From this standpoint it might be expected that glycogen would occur in at least some blood cells of various organisms, whether developing or mature.

### Neural and Hormonal Factors

In the vertebrate storage and mobilization of liver glycogen are greatly influenced by neural and hormonal factors. It has been demonstrated (*82, p. 190; 99, p. 675;* and *10, p. 938*) that puncture of the floor of the fourth ventricle of the vertebrate brain causes a decrease of liver glycogen, and that both the nervous and the hormonal systems are operative in the process. The autonomic nervous system is considered to be primarily involved (*99, p. 675*). It is also generally recognized (*82, pp. 139–142; 44, p. 849; 99, p. 680; 71, p. 829; 10, pp. 924 and 933*) that, at least under certain conditions, insulin tends to increase and adrenalin to decrease the glycogen reserves of the liver. Arthropods, including insects, have long been known to possess a sympathetic or stomatogastric nervous system (*45, pp. 448 and 518*). Like the vertebrate autonomic nervous system, it seems to be especially associated in a functional manner with such autonomic processes as cardiac and perhaps respiratory rhythms, and may possibly be related, either directly or indirectly, to such metabolic processes as hormone secretion and storage and mobilization of energy reserves in the tissues. That the insect nervous system is related to hormone secretion is indicated by a number of investigations (*35, 60, 65, 109, 110, 111, p. 40,* and papers cited in *90*).

Medvédéva (*73*), determining total reducing power, reported that hyperglycemia was produced in the silkworm by injection of adrenalin and to a less extent by injection of water. She attributed the water hyperglycemia to a mechanical stimulation of the vegetative nervous system by the needle puncture. She found that, although the adrenalinic hyperglycemia could be produced in the fifth instar, it could not be produced in the spinning larva, the chrysalis (at about the middle of the pupal period), or the adult. She attributed these differences to changes in the nervous system of the spinning larva and the pupa, to some unknown factor that assures glycogen storage in the spinning larva, and to a lack of glycogen reserve in the adult. Medvédéva obtained no insulin hypoglycemia by injecting insulin into the silkworm larva and adult, but did obtain a hyperglycemia in the larva, which he ascribed to mechanical stimulation of the nervous system. Ermakov (*31*) obtained an increase and a decrease of reducing power in silkworm blood by ligaturing the body of the larva in different regions and removing that portion not used for deter-

mination of reducing power of the blood. He considered the hyperglycemia to be due to disturbance of the larva's sympathetic nervous system but had no explanation for the hypoglycemia. Hemmingsen (*49*) injected insulin into larvae but obtained such variable results that no conclusion could be reached regarding the effect of this hormone on the insect's blood sugar.

Thus, observations and results reported in the literature suggest that in the insect hormones occur in relation to the nervous system, and that the insect's physiological mechanism, whereby it stores, mobilizes, and controls its glycogen reserves, probably involves both neural and hormonal components. It is obvious, however, that neural components are not directly involved in a control of blood-cell glycogen, even though they should directly affect other glycogen-containing tissue cells. Although insufficient data are available to show whether the blood-cell glycogen is under hormonal control, the occurrence in insects of hormonal control of or influence on blood-cell glycogen is a possibility.

### SIGNIFICANCE OF BLOOD-CELL GLYCOGEN IN THE DIFFERENT INSTARS

The results reported above and illustrated in figure 3 show that blood-cell glycogen increases during development to a maximum in the prepupa and then very rapidly falls to a low value in the early part of metamorphosis. These variations are in accordance with the results of Babers (*3*) and other investigators. Thus, whatever the causes of glycogen storage, the stored glycogen is utilized during metamorphosis. Since the blood-cell glycogen increases with increase of carbohydrate intake and decreases during starvation, indicating that conversion from and to glucose occurs, it seems more probable that the blood-cell glycogen is stored carbohydrate reserve than that it represents the transport of carbohydrate from the intestine during alimentation. Thus, one function of the blood cells appears to be to serve, like other tissue cells, for the storage of glycogen to be used during metamorphosis. Whether the glycogen is utilized by the blood cells themselves, as they play their roles during development, or whether their glycogen is made available as energy for other tissue cells is not evident from available data.

### KINDS OF BLOOD CELLS CONTAINING GLYCOGEN

The results reported here show that when sufficient glucose was ingested by larvae most of the blood cells contained glycogen inclusions. There seemed to be one cell type, however, in which glycogen inclusions were not observed which, in the preparations stained by the Bauer technique and counterstained with thionin, appeared as an irregularly outlined cell with a relatively small nucleus that seemed to consist of a number of chromatin granules (pl. 2, *A*, *i*, and *B*, *f*). These cells are here called "rhagmatocytes" in accordance with a tentative cell classification. The oenocytoids also were not observed to contain glycogen.

The plasmatocytes usually showed a greater tendency to contain glycogen than did the cystocytes, although in a few preparations the cystocytes were observed to contain glycogen when little or none was detected in the plasmatocytes. Of the several authors who have

reported the occurrence of blood-cell glycogen, Bogojavlensky (*14*) appears to be the only one who has attempted to identify the types of blood cells that contained the glycogen. In the walking stick *Carausius morosus*, and by implication in several other Orthoptera, Bogojavlensky found glycogen inclusions in spindle-shaped cells that were capable of becoming rounded, and that composed about 70 percent of the blood-cell population, but he did not state whether glycogen was found in the spindle-shaped cells, capable of becoming rounded, which comprised his second class of blood cells in *Bombyx mori* and by implication in several other Lepidoptera, and which would seem to correspond to the cells of *Prodenia eridania* here called plasmatocytes. Similarly, Bogojavlensky found glycogen in the proleucocytes of *C. morosus*, but not in those of *B. mori*. He also found a small amount of glycogen in his round cells with small nuclei, or micronucleocytes. He also reported that the granules of the granular leucocytes of *C. morosus* gave an iodophilic reaction with Lugol's solution and contained a carbohydrate (glycoproteid) component. Considering the fact that blood-cell glycogen is markedly influenced by carbohydrate absorption, the observations of Bogojavlensky and those reported here are not necessarily inconsistent, especially since there is not complete agreement with respect to insect blood-cell types and since *P. eridania* was used here and *B. mori* and other species were used by Bogojavlensky. The present observations and those of Bogojavlensky, that certain blood-cell types tend to contain more glycogen than other types, are in accordance with the observations (*4*, *21*, *29*, *42*) that the granular leucocytes but not the mononuclears of vertebrates contain glycogen.

### FORMS OF GLYCOGEN INCLUSIONS IN BLOOD CELLS

It has been shown that glycogen inclusions may have different forms in different kinds of blood cells. Thus, the cystocytes characteristically contain relatively small granular inclusions. Certain other cells may seem to be more or less diffusely impregnated with glycogen and also to contain relatively large round or nearly round inclusions. In accordance with a tentative blood-cell classification, these cells are here called spheroidocytes. The plasmatocytes characteristically contain smooth-contoured, round, ovoid, or somewhat irregular glycogen inclusions, which may differ greatly in size, even within a single cell. Bogojavlensky (*14*) described similar glycogen inclusions in the spindle-shaped blood cells of *Carausius morosus*, and the glycogen inclusions figured by Vaney and Maignon (*104*) seem to be of analogous forms. The glycogen inclusions in the vertebrate granulocytes and pus cells described by some of the authors already cited seem to be small and of fairly uniform size.

The observations that some of the glycogen inclusions may be faintly stained or stained deeply at their peripheries but only faintly or not at all in their centers can be variously interpreted. They suggest that glycogen might occur either uniformly throughout the inclusion or be locally concentrated at the periphery. In no inclusion did glycogen appear much more concentrated at the center than at the periphery. The plasmatocytes frequently contained colorless vacuoles of the same general aspect as the glycogen inclusions and, like the glycogen inclusions, were often located at the poles of the fusiform cells. Bogojavlensky (*14*) found polar vacuoles in spindle-shaped cells of *Carau-*

*sius morosus* when they were observed in vivo, and from comparison of them with Best and Lugol stained preparations he concluded that they contained glycogen. It would thus appear either that chromic acid fixation followed by the Bauer staining procedure incompletely fixes and stains some of the glycogen in the blood-cell inclusions, or that blood-cell glycogen might be stored in preexistent cytoplasmic vacuoles or might be mobilized from and leave behind glycogen-free cytoplasmic vacuoles. The fact that, even within a single plasmatocyte (from a glucose-fed larva), deeply stained glycogen inclusions may vary from tiny granules to approximately nuclear size indicates that glycogen may also appear in the cytoplasm without being associated with large preexistent vacuoles. Sometimes the large colorless vacuoles and glycogen inclusions were found to occur at the same time in a single blood cell.

### THE GLYCOGEN COUNT AND THE GLYCOGEN INDEX

With few exceptions the individual glycogen counts were of the same general form as the average counts shown in figure 1, *B* and *C*. The exceptional individual counts were similar in form to that represented by circles in figure 1, *E*, in that the number of cells in class 3 exceeded the number in class 3+. Examination of the smears corresponding to the exceptional counts showed that few or none of the cystocytes contained glycogen inclusions. Figure 1, *E*, also shows that class 3+ may exceed class 3 in number of cells because the cystocytes tend to enter class 3+ more readily than classes 1, 2, and 3. Figure 1, *D*, shows that as the glycogen index increases the number of cells in classes 1, 2, 3, and 3+ increases, but that the cells of class 1 increase relatively more than those of class 2, which increase relatively more than those of class 3.

It appears, therefore, that as the glycogen index increases two shifting processes occur in the glycogen count. First, cells may pass relatively slowly from class 0 through classes 1, 2, and 3 to class 3+. Such a process would tend to cause a shift of the maximum from class 0 to class 1, when the glycogen index becomes sufficiently high. This is shown by figure 1, *D*, to have actually occurred. Theoretically, the maximum should shift further from class 1 through classes 2 and 3 to class 3+, provided no limiting factors hinder the processes of glycogen accumulation in the blood cells as the glycogen index becomes still higher. It is probable, however, that limiting factors do exist in the insect and would prevent the maximum from shifting all the way to class 3+. Secondly, cells may pass very rapidly from class 0 through classes 1, 2, and 3 to 3+, or they may move from class 0 to 3+ without going through the other classes at all. Either process would cause a greater number of such cells to remain in class 3+ than in classes 1, 2, and 3. Figure 1, *A–E*, shows that actually class 3+ tends to be greater than class 3 in numbers of cells, and figure 1, *E*, indicates that, with regard to cystocytes, class 3+ exceeds classes 1 and 2 as well as class 3.

The microscopic observations also are in accordance with this interpretation of these figures. The cystocytes tend to contain glycogen inclusions in the form of a relatively large number of small granules, whereas the plasmatocytes tend to contain fewer glycogen inclusions of relatively large size (pl. 2, *C*; 5, *C* and *D*).

These results indicate that, in the glycogen count, as the index increases the plasmatocytes shift according to the first (slow) and the cystocytes according to the second (rapid) process.

### STATE OF THE BLOOD-CELL GLYCOGEN

The blood-cell glycogen reported here includes only that which is histochemically detectable, whereas that reported by Babers (*3*) includes that which is determined by chemical analysis. It should not be assumed that the histochemically and chemically determined forms of glycogen are identical (*4*, *76*). Bakker (*4*) found that chemically and histochemically detectable glycogen of exudate leucocytes may differ. Willstätter and Rohdewald (*113*) indicated that the glycogen of horse leucocytes exists mostly as a soluble lyoglycogen and to some extent as a less soluble, protein-bound desmoglycogen which, unlike the lyoglycogen, does not show the iodine staining reaction. They (*112*) also indicated that, when the leucocytes leave the circulating blood and migrate through the tissues, some conversion of desmoglycogen into lyoglycogen occurs. Bierry, Gouzon, and Magnan (*13*) reported that hepatic glycogen occurs as free (soluble) and protein-bound (insoluble) glycogen. Other evidence (*86*, *87*, *88*) also indicates the possibility of glycogen-protein combinations occurring in cells. Wajzer (*107*) found that, in the frog and the guinea pig, a large proportion of the liver glycogen normally occurs in combination with protein (proteid glycogen). Bogojavlensky (*15*) found that fat-body glycogen of the silkworm is much less soluble than the glycogen in other tissues. It is therefore quite possible that insect blood cells may contain lyo- and desmo- forms of glycogen, but distinction between them will depend upon future investigation.

### GLYCOGEN IN ALIMENTARY EPITHELIAL CELLS

The results reported here indicate that, in the normal course of development of *Prodenia eridania*, glycogen is absent from the midgut epithelial cells and the blood cells at the time of hatching, that it appears earlier in the gut cells than in the blood cells, that it occurs in gut cells in each instar, and that individual variations are large. This is in accordance with the results of Babers (*3*), who found no glycogen in the larva at the time of hatching. It cannot be concluded from these observations that the glycogen of the midgut epithelial cells is synthesized from ingested glucose as a part of the absorption process, for it is possible that these cells, like those of the blood and the other tissues, are able to store up carbohydrate reserves from glucose in the blood. The question of the occurrence of glycogen synthesis in the intestinal epithelia of vertebrates as a part of the mechanism of glucose absorption is as yet unsettled; in fact, it seems that it does not occur (*106*, *53*, *68*). Nevertheless, glycogen has been reported in the alimentary epithelium of the mammal by Rouget (*94*), Creighton (*22*), Von Fürth (*37*), and Horne and Magee (*53*); of the frog by Barfurth (*5*); of the fly larva (epithelium?) by Bernard (*8*); of the silkworm by Bogojavlensky (*15*) and Paillot (*78*, *79*); of *Limax* by Barfurth (*5*); of *Daphnia* by Von Dehn (*24*) and Beutler (Gallistel *39*); and of *Chironomus* larva by Harnisch

(*46*). Von Dehn (*24*) found gut-cell glycogen variable in *Daphnia* and, in general, detected it histochemically in animals that had been fed starch but not in starved animals. Gallistel (*39*) reported glycogen in the midgut cells of *Daphnia* only when there was an abundance of fat and glycogen in the fat-body cells. Bogojavlensky (*15*), making use of starvation and subsequent feeding procedures, reported that, after prolonged starvation of silkworms, glycogen remained only in the wall of the anterior part of the hindgut, the other tissues containing none, and that, with subsequent feeding, glycogen occurred first in the fat-body cells and later in the midgut epithelium. Vaney and Maignon (*104*) failed to find glycogen in the digestive epithelium of the silkworm, and De Boissezon (*16*) did not detect it in the epithelia of the midgut and hindgut of *Culex pipiens*.

From the information thus available it would seem that glycogen occurs generally, but variably, in the gut epithelia of animals, that the gut-cell glycogen varies with the intake of carbohydrate, and that it probably represents a mere storage of carbohydrate reserve rather than a glycogen synthesis that acts as a part of the glucose-absorption mechanism.

## GLYCOGEN IN OTHER TISSUES

The histochemical detection of glycogen in other tissues of insects has been reported as follows:

| *Tissue and insect* | *Investigator* |
|---|---|
| Fat body: | |
| Fly larva | Bernard (*9*). |
| Silkworm | Vaney and Maignon (*104*), Bogojavlensky (*15*), and Paillot (*78, 79*). |
| Tent caterpillar | Rudolfs (*96*). |
| Mosquito | De Boissezon (*16*). |
| *Chironomus* larva | Harnisch (*46*). |
| Oenocytes: | |
| Lepidoptera | Hollande (*51*). |
| Aquatic Hemiptera | Poisson (*85*). |
| Silkworm | Bogojavlensky (*15*) and Paillot (*79*). |
| Pericardial cells: | |
| *Gryllus campestris* and *Carabus auratus* L. (after injection of glucose) | Hollande (*52*). |
| Silkworm | Paillot (*79*). |
| Malpighian tube: | |
| Silkworm | Paillot (*79*). |
| *Chironomus* larva | Harnisch (*46*). |
| Reproductive system, silkworm | Bogojavlensky (*15*) and Paillot (*79*). |
| Eggs, etc | See literature cited by Babers (*3*). |
| Nervous system (ganglia), silkworm | Bogojavlensky (*15*). |
| Hypodermal cells, silkworm | Bogojavlensky (*15*) and Paillot (*78, 79*). |
| Muscle: | |
| Silkworm | Vaney and Maignon (*104*). |
| Tent caterpillar | Rudolfs (*96*). |
| Mosquito | De Boissezon (*16*). |
| Silkworm | Paillot (*79*). |
| *Chironomus* larva | Harnisch (*46*). |
| Associated with tracheal walls, silkworm | Bogojavlensky (*15*) and Paillot (*78, 79*). |

In the present investigation no polysaccharide was observed in the tracheal walls of *Prodenia eridania*. The ventral ganglia contained polysaccharide, and Bogojavlensky (*15*) made similar observations in the silkworm. The polysaccharide observed in the armyworm ganglia might or might not be located in the nervous tissue itself. Havet (*47*) reported that glycogen in the nerve centers of several animals, including *Limax*, is contained only in the neuroglial cells. Schabadasch (*97*) reported glycogen as occurring in both glial and neural cells in the mammal. Vaney and Maignon (*104*), Bogojavlensky (*15*), and Paillot (*79*) all found that striated muscles in the silkworm appeared to contain either no glycogen or a small amount spread diffusely throughout the substance of the fiber; but none of these authors fed large quantities of glucose to the insects. When *Prodenia eridania* larvae were fed large amounts of glucose, their striated muscles were found to be heavily loaded with polysaccharide, which appeared to be diffuse throughout the fiber but relatively concentrated at the cross striations; the alary muscle fibers, however, were relatively poor in polysaccharide. That histochemically detectable polysaccharide is definitely related to the cross striations of muscle in vertebrates is indicated in papers by Arnold (*1*), Creighton (*22*), Von Studnitz (*101*), Noll and Becker (*77*), and Feyel (*32*). Glycogen was also observed to occur in the labial glands of *Prodenia eridania*, but Vaney and Maignon (*104*) and Paillot (*79*) failed to detect it in these glands of the silkworm.

Since ingested glucose influences the polysaccharide content of tissues, disagreements of reported observations may be due in part to possible differences in food intake by the insects observed. These observations on *Prodenia eridania* indicate that many of the larva's tissues have the capacity to store up much more polysaccharide than they seem ordinarily called upon to store.

Although glycogen has been reported in the nuclei of some animal cells (*17*, *62*), polysaccharide has not been observed in the nuclei of any of the blood or other tissue cells of *Prodenia eridania*, insofar as the nuclear structures in these tissue smears could be distinguished.

### SITE OF GLYCOGEN SYNTHESIS

Bogojavlensky (*15*) reported that the fat body of the silkworm is the site of the first glycogen synthesis during poststarvation food intake. Babers (*3*) showed that, whereas the tissues of the normal sixth-instar *Prodenia eridania*, exclusive of the blood and alimentary tract, contained on an average about 4 percent of glycogen, dry weight, the isolated fat-body tissue contained about 23 percent. Although it is possible that glycogen synthesis may occur solely or chiefly in the fat body, whence it could be transported to other tissues, this does not seen probable, for Babers (*3*) found practically all the blood glycogen of this insect localized in the blood cells and almost none in the plasma. It is more likely, therefore, that the blood cells themselves possess a mechanism for gluconeogenesis and glycogenolysis.

### INFLUENCE OF DIFFERENT HISTOCHEMICAL PROCEDURES

Some difference of opinion has been expressed with regard to the adequacy of different fixatives used in the histochemical detection of

glycogen (*67, pp. 226–227*). Various procedures are recommended for use with insects. Brammertz (*17*) used Carnoy's fluid and Best's carmine stain (controlled by the iodine and salivary tests) to demonstrate glycogen in the embryos of *Apis mellifera* L. (*A. mellifica*),[7] *Bombyx mori*, and *Meloe proscarabaeus* L. (*M. carabaeus*). Bogojavlensky (*15*) fixed silkworm tissues in Carnoy's fluid and in saturated solution of corrosive sublimate, saturated with dextrose, and reported Carnoy's fluid satisfactory for glycogen fixation. De Boissezon (*16*) used absolute alcohol and Best's carmine to fix glycogen in *Culex pipiens* L. Gendre (*40*) modified Bouin's fixative by replacing the water with 95-percent alcohol saturated with picric acid, and reported the modified fluid satisfactory for glycogen fixation in silkworm tissues. Paillot reported Gendre's fixative as satisfactory for glycogen fixation in the fat body (*78*) and in the hypodermal cells (*79*) of the silkworm.

For glycogen detection in tissues of animals other than insects, Carnoy's fluid, absolute alcohol, and the fixatives of Pasteels and Léonard and of Gendre are recommended. Von Studnitz (*101*) used absolute alcohol and Lugol's solution to demonstrate glycogen in the frog's sartorius muscle. Noll and Becker (*77*) used absolute alcohol and Best's carmine to demonstrate glycogen in muscles of the rabbit and the hen. Gallistel (*39*), studying glycogen in *Daphnia magna* Straus, fixed glycogen chiefly with Carnoy's fluid but also with absolute alcohol and stained by Best's carmine method or the iodine method of Langhans. Havet (*47*), in a study of glycogen in the nervous systems of the rat, the cat, and *Limax*, fixed with absolute alcohol or 92-percent alcohol containing iodine, and stained by Best's method or by the iodine method of Prenant; he considered formol and Bouin's fixative unsatisfactory. Gendre (*40*), using his own fixative, reported satisfactory glycogen fixation in the dog, adult monkey, fetus of monkey, adult rat, newborn rat, and silkworms; he stained by methods of Lugol, Best, and Bauer. Coutelen and Cochet (*20*), in a study of glycogen in the larvae of cestodes, found absolute alcohol better than water fixatives, with or without picric acid, better than formol saturated with glucose or corrosive sublimate saturated with glucose, and better than the fixatives of Bouin, Zenker, Carnoy, Flemming, and Altman; Coutelen and Cochet used their own iodine staining technique. Feyel (*32*), in a study of glycogen in striated muscle fibers and liver of the frog, employed fixation in absolute alcohol with paraffin embedding, and also Bouin-Allen, Gendre, and Pasteels-Léonard's fixatives, each with dioxane-paraffin or celloidin embedding; she stained by the gum-iodine method, the iodine method of Langhans, Best's carmine method, and Bauer's method, and employed the salivary counterproof method. Feyel concluded that the best results were obtained by fixing the muscles for 20 to 24 hours in Bouin-Allen fixative and embedding in celloidin. Pasteels and Léonard (*81*) studied the demonstration of glycogen in embryonic tissue and in adult liver by fixing the tissues in absolute alcohol for 24 hours, in alcoholic formol

[7] Scientific names of insects in parentheses immediately following other scientific names are those appearing in the reference cited. Other scientific names are those currently in use according to the Division of Insect Identification of the Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture.

for 24 hours, in formol saturated with dextrose for 24 hours, in Carnoy's fluid for 4 hours, in Zenker's fluid for 24 hours, in the Bouin-Allen fixative for 6 hours, and in their own fixative; they stained by the methods of Lugol, Best, and Bauer, and employed the salivary counterproof. They concluded that the most satisfactory fixative for glycogen was an aqueous fixative saturated with picric acid, that the Bouin-Allen fixative was satisfactory for hepatic glycogen, that aqueous fixations were required for blastulae, gastrulae, etc., and that their own dioxane-picric acid-formalin-acetic acid liquid was satisfactory for fixation of glycogen.

Lison (*67, p. 230*) stated that the Best carmine method has no histochemical specificity. Lee (*64, p. 288*) recommended that preparations be made in tripilicate, to be used with the iodine method, Best's carmine method, and the salivary counterproof method. Bauer (*7*) reported that his method would selectively demonstrate polysaccharides such as glycogen, galactogen, starch, tunicin, and cellulose. Lison (*67, p. 232*) considered that, inasmuch as the coexistence of many polysaccharides in a given biological location is unlikely, Bauer's method is a good histochemical test for glycogen. Lee (*64, p. 289*) considered Bauer's method to be less specific than others. Giovannola (*41*) concluded that the polysaccharide reaction of Bauer is better than the Best stain for many kinds of protozoa. Gendre (*40*) considered that the iodine reaction, with salivary counterproof, is specific, that the Best method is less specific than the Bauer method, which itself is not very specific; he recommended that the Bauer method be controlled by the mucicarmine method of Mayer and the method of salivary counterproof. Coutelen and Cochet (*20*) stated that, among the glycogen methods they used, only the iodine method and the Bauer method possess histochemical value and that, as the latter is positive for other polysaccharides than glycogen, it should be accompanied by other methods. Bakker (*4*) obtained results indicating that positive and negative iodine reactions are insufficient evidence upon which to decide whether or not a cell contains glycogen.

In the present work the fluids of Gendre, Bouin, Bouin-Allen, Pasteels and Léonard, Neukirch, and Vastarini-Cresi gave unsatisfactory glycogen fixation when applied as indicated in table 1. These fluids were applied to blood and tissues of larvae that had been heat-fixed, and the blood and tissue smears were not subjected to the usual dehydrating and embedding procedures. None of the fixatives—absolute alcohol, Carnoy's fluid, and Zenker's fluid—that were found to give better results (table 1) contained picric acid.

The staining procedures used here were chiefly the method of Bauer, which was supplemented with Lugol's iodine method, the method of Best, and as counterproofs, Mayer's mucicarmine method and the method of salivary digestion, insofar as blood and midgut epithelial cells were concerned. The results are considered to indicate that the polysaccharide in the blood and the gut cells is glycogen, but since only the Bauer method was used on most of the other tissues, it cannot be concluded that the polysaccharide occurring in those tissues is glycogen. The results of this investigation, however, taken in conjunction with those reported by the other investigators, indicate that the observed tissue polysaccharide is probably glycogen.

## SUMMARY AND CONCLUSIONS

Observations on the occurrence of glycogen in blood cells and midgut epithelial cells, as well as of polysaccharide in various other tissues, of the southern armyworm (*Prodenia eridania* (Cram.)) are presented and discussed with reference to other literature bearing upon the subject. The results of several experimental procedures are also presented. From the observations and results reported here, the following conclusions may be drawn in regard to histochemically detectable glycogen or polysaccharide in the southern armyworm:

(1) Glycogen occurs normally in the blood cells, midgut epithelial cells, and certain other tissue cells of the larvae.

(2) Neither blood-cell glycogen nor midgut-epithelial-cell glycogen occurs normally in the larva at the time of hatching.

(3) As indicated by values of the glycogen index, blood-cell glycogen increases during normal larval development until it attains a maximum in the prepupa; after pupation it rapidly decreases and remains at a low level during most of the pupal period, tending to disappear toward the end of the pupal period. In the first instar, and probably in the adult, blood-cell glycogen occurs very infrequently.

(4) When newly hatched larvae are kept without food, neither blood-cell nor midgut-epithelial-cell glycogen occurs up to the time of death; but when they are given food containing sufficient carbohydrate, particularly glucose, midgut-epithelial-cell glycogen appears. It tends to appear sooner than blood-cell glycogen, which occurs infrequently.

(5) In sixth instars blood-cell glycogen decreases or disappears during starvation and increases after ingestion of food containing carbohydrate, particularly glucose.

(6) As many as 85 percent of the larva's blood cells may contain glycogen inclusions as a result of ingestion of sufficient carbohydrate, particularly glucose.

(7) Most, but apparently not all, of the insect's blood-cell types can contain glycogen inclusions as a result of glucose ingestion.

(8) The blood cells here called plasmatocytes and cystocytes showed a greater tendency to contain glycogen inclusions than did the other blood-cell types. When the glycogen index is sufficiently high, glycogen inclusions may appear, however, in the cells here called spheroidocytes, polypodocytes, and nematocytes, as well as in some others. Glycogen inclusions may also occur in cells undergoing mitotic division.

(9) The glycogen inclusions tend to be fewer and larger in the plasmatocytes than in the cystocytes.

(10) The glycogen count of the normal insect typically has the form $0>1>2>3<3+$, where the numbers refer to the classes in the count.

(11) As the glycogen index increases, the cells decrease in class 0 and increase in the other classes. The increase is greater in 1 than in 2 and greater in 2 and 3+ than in 3. When the glycogen index increases enough, the maximum shifts from class 0 to class 1.

(12) The plasmatocytes tend to pass through all the classes from 0 to 3+ relatively slowly, whereas the cystocytes tend either to pass through them rapidly or to enter class 3+ without passing through 1, 2, or 3 at all.

(13) Average glycogen counts from nonligatured larvae and from the fore and hind ends of ligatured larvae have similar forms.

(14) Glycogen may occur in midgut epithelial cells in each instar.

(15) Midgut-epithelial-cell glycogen is influenced by food ingestion, decreasing as a result of starvation and increasing after high intake of carbohydrate, especially glucose.

(16) Glycogen inclusions can occur in the labial-gland cells and the oenocytes of first instars.

(17) Polysaccharide can also occur in the following tissues of larvae fed sufficiently large amounts of glucose-cornstarch-turnip leaf food: Ganglia (and connectives) of the ventral nerve cord, labial glands, Malpighian tubes, fat body, oenocytes, gonads, pericardial cells, integument, walls of the foregut and hindgut, striated muscle fibers of body muscles, gut musculature, cardiac muscle, and the alary muscle fibers.

(18) In the striated muscle fibers the polysaccharide occurs diffusely in the fibers and concentrated at the cross striations.

The observations and results reported here, taken in conjunction with the other results reported in the literature cited in this paper, tend to support the following conclusions:

(1) *Prodenia eridania* is not exceptional in that some of its circulating blood cells normally contain glycogen inclusions.

(2) The blood-cell glycogen represents a store of reserve carbohydrate rather than, for example, a newly absorbed foodstuff that is being transported by the blood to the tissues; hence, storage of reserve carbohydrate may be recognized as one function of the southern armyworm's blood cells, particularly the plasmatocytes and the cystocytes.

(3) The glycogen-containing blood cells themselves possess mechanisms for gluconeogenesis and glycogenolysis.

(4) Gluconeogenesis and glycogenolysis may be influenced by a number of different factors, which are discussed.

(5) The midgut-cell glycogen indicates a storage of reserve carbohydrate rather than a glucose-absorbing mechanism.

(6) Glycogen synthesis occurs in those tissues in which the glycogen appears, rather than exclusively in a single particular tissue, such as the fat body, although this body may synthesize and store a relatively large amount of glycogen.

(7) Glycogen probably occurs in the glycogen-storing cells in both a soluble and a relatively insoluble form.

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# "PARTICLES" AND THEIR RELATIONSHIP TO THE STRUCTURE OF ANIMAL FIBERS [1]

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## INTRODUCTION

Many of the problems concerned with the variation in the qualities of animal fibers can never be solved until a more comprehensive understanding of the minute structure of the fibers has been achieved. Of the many animal fibers, wool is the most widely used. The peculiar properties of wool have led to much speculation as to the structure of the fibers. More than a century ago Thomas Plint, a woolen manufacturer, made the following statement, as quoted by Youatt (*24, p. 84*):[2]

> Respecting the application of the microscope to the examination of the fibre, I am decidedly of opinion that a careful and minute examination of wools differing in their felting properties would issue in the detection of some specific difference of structure. This property is altogether inexplicable, at least in my mind, except on the supposition that the extreme surface of the fibre is irregularly *feathered,* and that, by compression, these feathered edges become entangled and locked together. These feathers must also point in one direction, viz, from the root to the extremity of the fibre; and if we suppose the feathered edge, or, more properly speaking, the individual tooth or feather, to be of a firm texture, it is evident that one tooth being pushed into another, would fasten like a wedge; and if we further suppose that the tooth or feather has a barb, similar to that on a whale-harpoon, the phenomena of felting are explained.

Plint's observation of the serrated edges of wool is also described by Youatt (*24, p. 87*), as follows:

> A fibre was taken from a Merino fleece of three years' growth * * *. It was taken without selection, and placed on the frame to be examined as a transparent object. A power of 300 (linear) was used, and the lamp was of the common flat-wicked kind. The focus was readily found; there was no trouble in the adjustment of the microscope, and, after Mr. Powell [the maker of the microscope], Mr. Plint had the first perfect ocular demonstration of the irregularities in the surface of wool—the palpable proof of the cause of the most valuable of its properties—its disposition to felt.

During the last century the larger elements of the structure of wool fibers were definitely established. The three main regions are the cuticle, cortex, and medulla. The cuticle is the layer of thin, horny, irregularly shaped, platelike cells that envelop the fiber. These cells may overlap each other or surround the fiber, but their edges always point toward its tip. Many patterns are formed on the surface of wool fibers by the arrangement of these cells. They differ in different animals; in fact, they vary from the root to the tip end on many fibers. The cortex, which lies just below the cuticle, constitutes most of the remaining portion of a wool fiber and sometimes all of it. It is made up of spindle-shaped cells that are intimately

[1] Received for publication March 12, 1941.
[2] Italic numbers in parentheses refer to Literature Cited, p. 302.

bound together, probably by some sort of binding substance. The strength and elasticity of wool are attributed largely to these horny cells. Many fine fibers are composed of only the cuticle and the cortex; however, in most wool fibers there is a central corelike structure, the medulla. It may differ in appearance in different kinds of wool.

Nathusius (*14*) disintegrated wool fibers by chemical means, employing sulfuric acid, alkali, or ammonia to separate the cells. He found the cuticle, cortex, and medulla to be composed of cells of different shapes. McMurtrie (*10*), employing the same reagents, examined these cells in detail with special reference to differences among various breeds of sheep. Walther's (*22*) ambitious measurements of 20,000 cortical cells, separated by sulfuric acid, showed that there are slight differences in size of wool-fiber cells among different breeds of sheep but they are not of sufficient magnitude to warrant definite conclusions.

Since disintegration of the fibers by chemicals always produces some swelling, attention was turned to biological methods of disintegrating fibers. Meunier, Chambard, and Comte (*11*) successfully employed an enzyme, pancreatin, for the separation of the cortical cells, and also of the cuticular cells. Waters (*23*) isolated the bacterium that causes pink rot of wool, and this organism was used by Gabriel (*6*) to break up the fibers for the study of the cellular characters of wool from Merino, Romney, and Lincoln sheep. Burgess (*2*) disintegrated wool fibers by the action of the enzymes trypsin or pepsin. The results were similar to those obtained with the proteolytic bacteria but were achieved much more rapidly.

Although the early workers thought that the cell represented the ultimate element of structure of animal fibers, later studies have disclosed smaller features. Striations lengthwise of the fibers have been noticed for a long time. Waldeyer (*21*), in examining hairs, concluded that these were fibrils ("Hornfibrillen"), which were constituent parts of cells, but were longer than individual cells. He believed them to be the elements of the structure of fibers, and he thought that they were produced by the cell protoplasm. Nathusius (*15*) confirmed the finding of these fibrils and their importance as structural units of the cortical cells but did not find substantiation of Waldeyer's (*21*) claims that the fibrils were larger than entire cells. Moreover, he also mentions an interfibrillar cementing substance.

Physicists and chemists are at present approaching the study of fiber structure from entirely different angles. Thus Astbury (*1*) is determining the shapes and the dimensions of the molecules and the way they fit together. Speakman (*19*), Harris and Rutherford (*8*), and others are explaining characteristic properties of wool in terms of chemical differences.

In the opinion of the writers, there is a large gap between the fields in which work has been in progress on wool. On the one hand, the microscopists examine wool at 300 to 500 diameters' magnification, usually to understand its gross structure, whereas the physicists and chemists study the size, shape, linkages, and other characteristics of the structural components of fibers in terms of angstroms (0.0001$\mu$). However, exact knowledge of the smallest visible units of structure of the fibers and the manner in which they are held together is yet very meager. Indications of the existence of particles in wool, mohair,

human hair, cattle hair, and hog bristles were obtained by Hardy and reported by Mohler (*12, p. 26*). Questions arise as to what may be revealed concerning the structure of the cuticle, the cortex, and the medulla when they are examined at higher magnifications than have ordinarily been used, and also whether details can be more sharply defined by special attention to optical and histological techniques at all magnifications. To obtain information on these points, the study reported in this paper was conducted from 1938 to 1940 at the United States Department of Agriculture, Beltsville Research Center, Beltsville, Md.

## EXPERIMENTAL METHODS

In seeking units of structure of animal fibers smaller than the fibrils reported by Waldeyer (*21*), wool fibers from Lincoln and Karakul sheep were chosen as the material for investigation. Several methods were employed to disintegrate the fibers in order to avoid artifacts. Mechanical disintegration was accomplished in two ways: (1) The fibers were dissected under the microscope (Greenough type), bits of the fiber being peeled off the surface with a sharp knife, and mounts made of these surface or cuticular scrapings and also of scrapings of the cortex; (2) fragments of fibers in a more finely divided state were obtained by grinding the sample in a Wiley mill and removing the coarser fragments by sifting the ground material through a fine-mesh glass cloth.

Chemical disintegration of the cells was obtained by a procedure similar to that followed by earlier investigators. This consisted in immersing mounted fibers in a warm solution of equal parts of sulfuric acid and water or 5-percent sodium hydroxide. Two or three fibers were mounted on a glass slide with adhesive tape, the fibers extending nearly the entire length of the slide. As the fibers disintegrated, successive segments were removed from the slide and mounted in glycerin. Slight pressure was applied to separate the fiber cells. In this manner slides were obtained that showed the various stages in the separation of the cells. Saturated aqueous solution of bromine, as well as perhydrol, was found to be satisfactory for disintegrating the fibers.

Finally, a new biological method of disintegrating the fibers was devised by the senior author, as reported by Mohler (*12*). It consists in feeding wool to carpet beetles,[3] *Anthrenus vorax* and *Attagenus piceus*, under controlled conditions. The discovery of this method resulted from an inspection of guinea-pig-hair samples that had been stored in large gelatin capsules. The snow-white hair had somehow been replaced by a snow-white powder; black and white hair by a gray powder. Closer inspection revealed a small hole in each capsule; the larvae of some insect had eaten through the gelatin of the capsules, devoured the hairs, and made their exit the same way that they had come. The entire sample cabinet proved to be infested with carpet beetles. It seemed logical to assume that if the beetles had lived only on the hair, their excreta would contain remains of fibers in a very finely divided state. Microscopical examination confirmed this. This lead was followed up by an experiment. Carpet beetle larvae were

[3] These were obtained through the courtesy of Ernest A. Back, of the Bureau of Entomology and Plant Quarantine, and of William A. Davidson, formerly of the Food and Drug Administration, U. S. Department of Agriculture.

placed in contact with various kinds of animal fibers, including several kinds of wool, hog bristles, and the extremely tough bristles from the tail of the African elephant. All of these were consumed with apparently equal ease and relish by the carpet beetle larvae. Passing through the digestive tracts of the insects, these animal fibers were reduced to a powder.

In view of this finding, the following experiment was performed: Fifty of the beetle larvae were put into each of a series of specimen jars having a capacity of about 25 cc. and provided with screw caps. A sample of wool was added to each jar, which was then set in a dark place at room temperature. In a few days the wool was reduced to powder by passing through the digestive tracts of the beetles.

To determine the arrangement of the particles in the fibers, cross sections and longitudinal sections were cut on a rotary microtome.[4] The fibers were prepared for sectioning by putting them through three changes of dioxane and then keeping them in a paraffin oven for 2½ hours in a mixture of one-third dioxane and two-thirds soft paraffin. At the end of that time they were transferred to hard paraffin (56°–58° C.), left for 2½ hours, and then embedded. The best success in cutting the sections was found with blocks that had stood about 1 week before cross sectioning.

Mounted specimens derived by the above methods were examined under different microscopic conditions, including ordinary transmitted light, oblique illumination, and polarized light. It was not practical to measure directly the small individual units found. However, by the use of an ocular step micrometer it was possible to observe chains of the minute structural units of the fibrils and to measure the length of several chains, from which the approximate size of individual units could be determined.

## EXPERIMENTAL RESULTS

Both the scrapings of the fibers and the fibers that had been ground and passed through the fine-mesh glass cloth were found to be too

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EXPLANATORY LEGEND FOR PLATE 1

*A*, Aggregates of particles with cementing material in a finely ground Lincoln fiber; mounted in acryloid.

*B*, Aggregates of particles in a finely ground Lincoln fiber; mounted in polystyrene.

*C*, Individual particles in finely ground Karakul fibers; mounted in polystyrene.

*D*, Individual particles in excreta of carpet beetles fed Lincoln fibers; mounted in polystyrene.

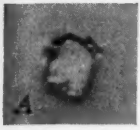
*E*, Striated appearance of an untreated Karakul fiber, as revealed by an impression on a transparent thermoplastic film.

*F–I*, Progressive stages in the disintegration of fibers through the use of sodium hydroxide: *F*, fiber swollen, chains of black particles visible in lighter edges of Karakul fiber; *G*, Karakul fiber greatly swollen, particles evident in medulla (corelike center); *H*, partial disintegration of Karakul fiber, medulla still preserved, cells broken down into fibrils; *I*, complete disintegration of Lincoln fiber, fibrils separated.

*J*, Cross section, about 5$\mu$ thick, of Karakul fiber, showing aggregates of particles.

*K* and *L*, Longitudinal sections of Karakul fibers, showing regular alignment of chains of particles. (*A–D*, × 3600; *E–L*, × 600)

[4] These sections were prepared by H. Dean Ray, of the Bureau of Animal Industry.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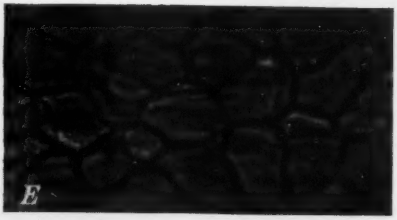
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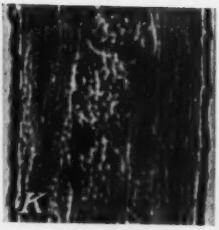
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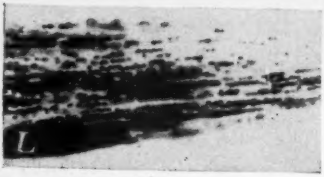
L

large to show the details clearly. However, the fine dust sifted from the ground fibers was satisfactory. On examination of this powder under the microscope with oblique illumination, aggregates (pl. 1, *A* and *B*) and individual small spherical units (*C*) could be seen. These individual units, for which the authors propose the name "particles," were only a fraction of a micron in diameter.

The arrangement of the cells, fibrils, and particles was best observed in the specimens that had been prepared by chemical treatment. Plate 1, *E*, shows an impression of an untreated Karakul fiber on a transparent thermoplastic film, obtained by a method previously developed by the authors (*7*). The somewhat striated appearance is evidently caused by fibrils, which in turn are composed of chains of particles. *F*, *G*, *H*, and *I* show progressive stages in the chemical disintegration of fibers. In *F*, these chains of black particles are visible in the lighter edges of the fiber. In *G*, which shows a greatly swollen fiber, the particles may be clearly seen in the medulla. In *H*, the fiber is partly disintegrated although the medulla is still preserved. The cortical cells have broken down into fibrils, in which the chains of particles hold together in a colloidal mass. In *I*, the fibrils have become separated during severe swelling. Here, again, the particles are seen to be arranged in single rows of chains. In the cortex the cells appear in parallel chains, whereas in the medulla the chains are irregular and very much twisted. It is possible that these chains of particles are not in the same plane for their entire length and may, therefore, be longer than they appear. The arrangement of these particles in single rows of chains and the approximately parallel alignment of many of the fibrils with respect to the length of the fiber, may be of fundamental significance in determining some of the important mechanical properties of animal fibers.

Inspection of the fine powder to which the wool was reduced by passing through the digestive tract of the carpet beetles revealed that it was made up of aggregates of many small pellets. On crushing, these pellets were found to be composed of small spherical units that appeared to be the same as those obtained by the other methods.

Examination of parts of fibers from Karakul sheep by ordinary transmitted light greatly facilitated the recognition of the particles because of the presence of pigment in many of them (pl. 1, *J*, *K*, *L*). The significant fact is thereby established that the individual particles may contain pigment, as shown also in *C*. The pigmented particles are usually black but some brown ones have been seen. It is not uncommon for black particles and white particles to exist in close proximity in the same fiber, but in any one fibril particles of one color appear to predominate (*K*).

In plate 1, *J*, which shows a thin cross section of a Karakul fiber, particles may be seen in aggregates of different shapes and sizes. In *K* and *L*, which show longitudinal sections of Karakul fibers, chains of particles are aligned in a regular manner.

Under polarized light this alignment is further manifested. A whole chain exhibits the same characteristics of double refraction as do the individual particles. The fibrils are bright when placed in a position 45° to the planes of vibration of the Nicol prisms but are extinct when placed parallel to the planes of vibration. When the "first order red" plate is inserted, the chains are alternately blue and yellow as they are rotated through the quadrants. Since the particles

are spherical, such behavior of the fibrils indicates that the particles are arranged in accordance with the alignment of submicroscopic internal structures.

Measurements of chains of particles yielded an estimated diameter of 0.6$\mu$ for individual particles. Particles obtained from finely ground Karakul fiber are shown in plate 1, *C*, and particles from the excreta of carpet beetles fed Lincoln wool fibers are shown in figure *D*, both mounted in polystyrene. A transparent film was observed repeatedly. Such films may be seen in pieces of finely ground Lincoln wool fiber mounted in acryloid (*A*). The particles are apparently attached to this film, which may therefore prove to be of the nature of a cementing material.

## DISCUSSION

The particles are apparently composed of keratin. The question of the relation of these keratin particles to micelles and crystallites naturally arises. For many years scientists have attempted to gain an understanding of the smallest structural units. What they have seen and their interpretations have given rise to controversies.

The definition of micelles has undergone several revisions through the years. Nägeli's conception, as reported by Nägeli and Schwendener (*13*), was that molecules are grouped into submicroscopical, that is, invisible, crystals which he termed "micelles." Speakman still refers to micelles in wool 200 angstroms thick and probably 10 times as long. Frey-Wyssling (*5*) modified the concept of micelles to apply to all the long molecular chains which are the framework that lends rigidity to a gel. This is an extension of thought in that it includes not only the aggregates of chain molecules in sufficient numbers to produce crystalline effects, but also those composed of but few parallel chain molecules. On this basis he proposed a theoretical structure of fibers which he believed would reconcile the two conflicting points of view: on the one hand, the existence of a bricklike structure composed of a disperse phase in a dispersions medium, such as conceived by Nägeli (*13*); and on the other hand, the existence of only a continuous structure interpreted by Sponsler (*20*) and Astbury (*1*) in X-ray methods. Frey-Wyssling (*5*) proposed a reticular fibrillar system of structure in which long molecular chains crystallize together in some regions, and in others are not sufficiently close or regularly parallel to form a crystalline lattice. There is no corpuscular disperse phase, but both micellar and intermicellar systems are continuous, penetrating each other. Individual micelles do not exist because they are all connected with one another and grow together; the micelles of Nägeli corresponded merely to crystalline regions within a continuous, porous structure. Farr and Sisson (*4*) emphasize that the possibility of further subdivision of cellulose particles into smaller, possibly submicroscopic, crystalline masses is not obviated by their conception of membrane structure. It is likewise possible that the keratin particles may in turn be composed of submicroscopic units of structure.

All the investigators mentioned studied cellulosic fibers except Astbury (*1*), who investigated fibers of animal origin. Further analysis in the several related fields will be necessary before the exact structures of fibers and their relationships are definitely established.

The particles in wool and other animal fibers are believed to be composed of keratin since their resistance to the action of chemicals is similar to that of the entire keratinous fiber. Furthermore, like the whole fiber, the particles are doubly refractive, as are the fibrils in which they occur. These particles are spherical, having a diameter estimated at approximately 0.6$\mu$. Nathusius (*15*), measuring dry fibrils by projection, although obtaining measurements between 0.3$\mu$ and 0.7$\mu$, concluded that no difference in the thickness of the fibrils could be demonstrated between different kinds of hairs or between wools of different fineness. The keratin particles appear to be uniform in size, although this point is difficult to determine at the high magnifications that must be employed to see them. In different mounting media their apparent size is slightly different, as demonstrated in plate 1, *A* and *B*. However, conclusive studies along this line have not been made. The particles have a linear arrangement, producing the fibrils that have been previously recognized.

A distinctive quality of the keratin particles is their capacity to bear pigment. As early as 1867 Kölliker (*9*) called attention to the presence of granular pigment in hair. In 1894 Nathusius (*15*) discovered that the pigment granules are present in the fibrils and emphasized that they are definitely oriented structures, not mere granular deposits of pigment.

A thin transparent film has been repeatedly observed in the present investigations (pl. 1, *A*). This may be a cementing substance which holds the particles and the chains together. Moreover, the presence of such a substance between the particles might be deduced from the fact that the particles pass through the digestive tract of the carpet beetle larvae apparently without any change. Consequently, the nourishment derived from the fibers by the larvae may well be from such a cementing substance. The presence of a cementing material has long been postulated by those investigators who broke up animal fibers into their constituent cells—Nathusius (*14*, *15*), Waldeyer (*21*), and Meunier and coworkers (*11*). Waldeyer (*21*) supposed that the fibrils were differentiation products of the cellular protoplasm and that a remnant of the protoplasm was left between the fibrils as an interfibrillar cementing substance.

The presence of cellulose particles having many properties similar to the keratin particles has been reported in plant cell walls by Farr and Eckerson (*3*). More than a century ago Schleiden (*16*) and Schwann (*18*) formulated their famous cell theory, declaring the fundamental similarity of cellular structure in the plant and animal kingdoms. About 20 years later Schultze (*17*) established the protoplasm doctrine, wherein it was recognized that protoplasm is similar in all living organisms. The present writers advance the possibility that particles are the smallest visible units of mechanical structures in both plants and animals.

## SUMMARY

To gain a clearer insight into the microscopical structure of animal fibers, research was carried on at the United States Department of Agriculture, Beltsville Research Center, Beltsville, Md., during the years 1938 to 1940.

Minute structural units, which the writers propose to call "particles," were observed equally well in fibers subjected to the following treatments: Disintegration by chemical means; reduction to a powder by a new biological method, namely, through digestion by carpet beetles; and grinding through a special mill and using only the resulting fine dust.

The particles are composed apparently of keratin, are spherical, uniform in size, and measure approximately 0.6$\mu$. They are doubly refractive to polarized light and sometimes bear pigment. Individual particles have been isolated and photographed.

The particles are aligned in chains, which constitute the fibrils. The chains lie parallel to the long axis of the fibers in the cortex, but they appear to twist and interlock in the medulla.

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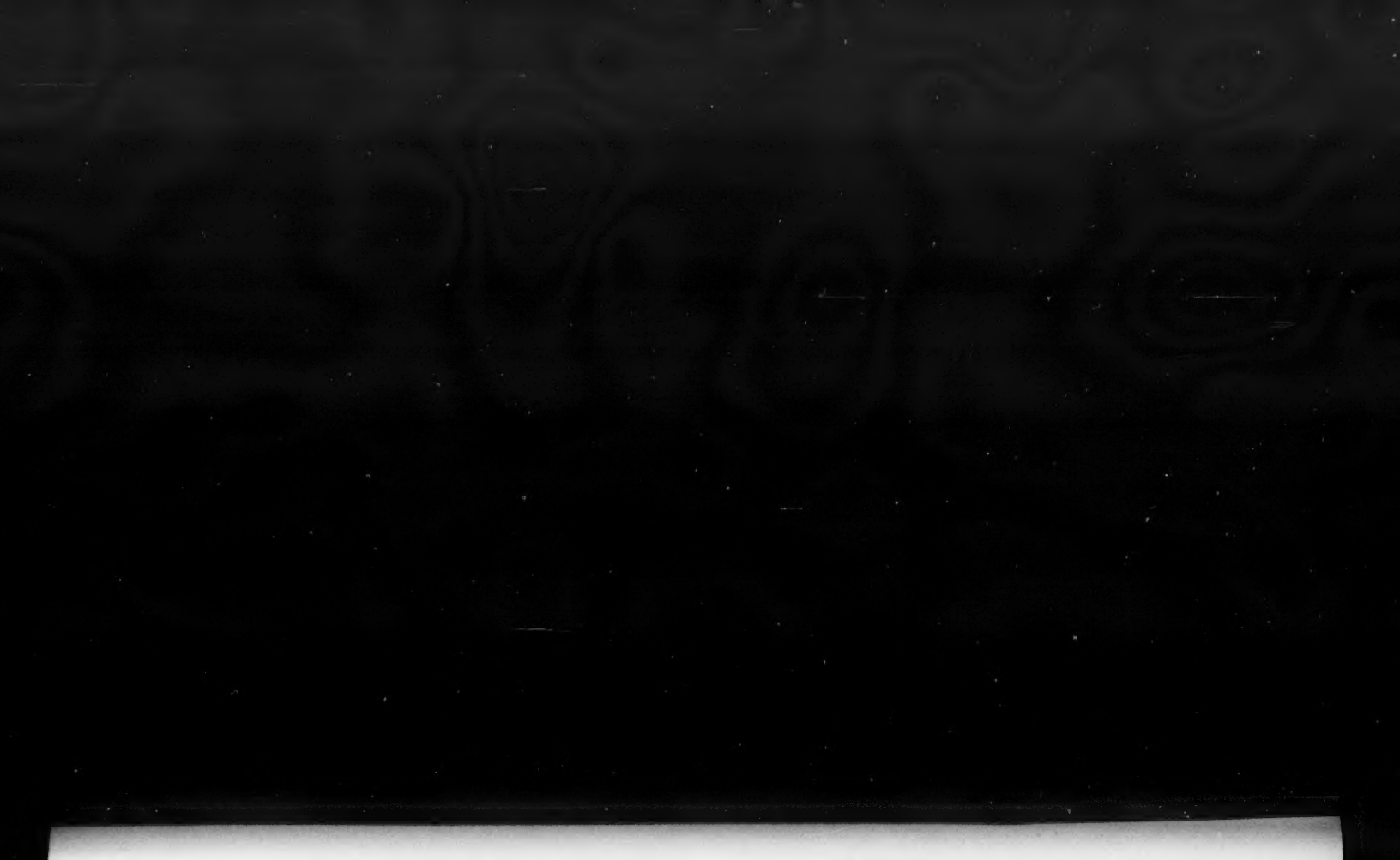
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